REMARKS

Claims 17, 19, 20, 21, 23, 24, 28, 29, 31, 33, 38, 41, 43, 49, 56, 59, 60, 61, 62 and 63 are pending. No new matter has been entered.

Priority

The Examiner has requested that Applicant specify basis in the provisional application (US Patent Application No. 60/115,125) for: blood samples that have not been fractionated into cell types; analysis of two or more genes for the same disease; quantifying a level of differential expression; and quantifying levels of RNA in samples.

Applicant submits that there is support for the claims in the provisional application. Applicant wishes to point out that the limitation of "blood samples that have not been fractionated into cell types" finds inherent basis in the provisional application, for example, at page 3, paragraph starting at line 12, which refers to "whole blood" and a "simple blood sample" which are inherently samples that have not been fractionated into cell types. Applicant wishes to point out that "analysis of two or more genes for the same disease" finds inherent basis in the provisional application, for example, at page 12, paragraph starting at line 3, which refers to analysis of genes ("gene(s)", e.g. p. 12, lines, 5 and 18), i.e. to analysis of two or more genes, for disease diagnosis (e.g. p. 12, line 14), where the analysis is in "a subject blood sample" (p. 12, lines 6-7). Applicant submits that these passages taken as a whole provide inherent basis for analysis of two or more genes for the same disease. Applicant wishes to point out that "quantifying a level of differential expression" and "quantifying levels of RNA in samples" find inherent basis in the provisional application, for example, at page 13 which recites "analysis is performed by quantitation and the difference is... increased amounts of the subject RT-PCR product(s) relative to the control RT-PCR product(s) and decreased amounts of the subject RT-PCR product(s) relative to the control RT-PCR product(s).", and at page 18, paragraph starting at line 4, which refers to RT-PCR analysis of "samples".

Claim Rejections – 35 USC § 103(a)

Claims 17, 19, 20, 21, 23, 24, 28, 29, 31, 33, 34, 38, 41, 43, 49, 56, 61, 62,and 63 are rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* (WO 98/49342) in view of Ralph *et al.* (US 6109857 and WO 98/24953).

Specifically, the Office Action contends that Sharma et al. teaches a method for identifying a marker useful for diagnosing a disease involving detecting and quantifying RNA in an unfractionated sample of whole blood from one or more subjects having the disease, where isolation of RNA is taught at p. 35, section 5.1.1. The Office Action further contends that Sharma et al. teaches that known differential display methods may be used to identify markers for the diagnostic probe patterns. The Office Action concedes that Sharma *et al.* does not teach use of an oligonucleotide of predetermined sequence such as primers specific only for RNA and/or cDNA complementary thereto. With regard to Ralph *et al.*, the Office Action contends that this reference teaches a similar differential display method to identify markers of disease in blood and confirmation of the differential expression using RT-PCR. The Office Action further contends that Ralph et al. teaches that mRNAs identified by RNA fingerprinting or differential display as being differentially regulated frequently turn out not to be so when examined by independent means, and that it is critical that the differential expression of all mRNAs identified by RNA fingerprinting be confirmed by an independent methodology. The Office Action concludes that it would have been obvious to one of ordinary skill in the art at the time the invention was made to have modified the methods taught by Sharma et al. so as to have included the RT-PCR step using oligonucleotides of predetermined sequence as taught by Ralph et al. so as to have provided a means to confirm the differential expression of the identified markers within a complete method of identifying two or more markers useful for diagnosing a disease. The Office Action additionally contends at page 15 that there is no evidence on the record to suggest that the teachings of Ralph *et al.* and Sharma *et al.* et al. are not valid, based on the Office Action's position that Ralph *et al.* and Sharma *et al.* are considered skilled in their fields, and that they set forth their teachings so clearly as to enjoy a presumption of validity.

Applicant respectfully traverses the rejections.

In particular, Applicant submits that the ordinarily skilled artisan would not be motivated to combine the cited teachings of Sharma *et al.* with those of Ralph *et al.* so as to arrive at the claimed invention, as contended by the Office Action since it can be clearly shown, as described below, that the ordinarily skilled artisan would not consider the cited combination of teachings to enable the claimed invention. Furthermore, Applicant submits that the ordinarily skilled artisan would not have a reasonable expectation of success in practicing the claimed invention when combining the cited teachings according to the Office Action's contention. Additionally, Applicant submits that the cited combination fails to teach all of the limitations of the claims.

In particular, Applicant submits that a pivotal contention of the Office Action upon which the rejections are based, i.e. the contention that Sharma *et al.* teaches, at page 35, section 5.1.1 a method for isolation of mRNA from blood samples which have not been fractionated into cell types, as required by the claims, would not be considered reasonably enabling by the ordinarily skilled artisan such that combination of this teaching with the cited teachings of Ralph *et al.* could be used to achieve detection of RNA encoded by a gene in RNA of a blood sample which has not been fractionated into cell types, as further required by the claims.

Applicant submits that the ordinarily skilled artisan at the time of the invention would not consider the cited teachings of Sharma *et al.* concerning a method for isolation of mRNA from blood samples which have not been fractionated into cell types at page 35, section 5.1.1, when combined with the cited teachings of Ralph *et al.*, to enable the claimed method in view of the directly relevant strong counter-teachings in the art described below, as strongly supported by the publication history of the inventors of Sharma *et al.* after the filing date of Sharma *et al.* directly relating to the teachings of Sharma *et al.*, and consistent with the total lack of any reduction to practice in Sharma *et al.* regarding any aspect of RNA expression in blood of any human or animal.

In particular, Sharma *et al.* teaches at section 5.1.1 isolation of RNA from human whole blood RNA for detection of disease-specific differential gene expression therein performed using a method plainly consisting of the following initial steps:

1) simple freezing of whole blood samples, i.e. without teaching addition of any type of stabilizing agent at this stage, including any cell-membrane stabilizing agent to prevent freezing-induced cell rupture or any ribonuclease inhibitor to prevent ribonuclease-induced RNA degradation (such that this step would therefore result in cell rupture induced co-release of RNA and abundant ribonucleases and concomitant ribonuclease-induced RNA degradation);

2) simple thawing of the frozen blood samples, i.e. without teaching addition of any type of stabilizing agent at this stage either, including any ribonuclease inhibitor (such that this step would therefore result in further co-release of RNA and abundant ribonucleases from the ruptured cells and concomitantly accelerated ribonuclease-induced RNA degradation in liquid phase);

3) simple centrifugation of the thawed blood samples for 5 minutes (i.e. without teaching addition of any type of stabilizing agent, including any ribonuclease inhibitor, during this prolonged liquid-phase stage, such that this step would therefore result in still further and extensive co-release of RNA and abundant ribonucleases from the ruptured cells and concomitant extensive ribonuclease-induced RNA degradation); and

4) lysis of samples in "*Solution A (4M Guanidine thiocyanate, 25mM Na-citrate, pH 7.0, 0.5%(w/v) N-laurylsarcosine, 0.1M 2-Mercaptethanol)*" (i.e. such that this step consists of addition of ribonuclease inhibitors to the samples only after the freeze-thaw cycle).

It can be clearly shown from the literature of the prior art that the ordinarily skilled artisan would expect that the blood freeze-thaw based methodology taught by Sharma *et al.* would result in such extensive degradation of RNA that the resulting RNA preparation would be unsuitable for RT-PCR analysis, particularly for sensitive applications requiring not only simple detection of RNA encoded by gene as required by the claims and as allegedly taught by Ralph *et*

al., but moreover requiring differential quantification of RNA encoded by the gene, as further allegedly taught by Ralph *et al.*, and as further required by the claims.

Namely, Kephart (Promega Notes Magazine Number 62, 1997; alternatively referred to herein as "Kephart"; cited by the Office Action dated 08/11/2006 as relevant prior art to the claimed invention) plainly experimentally demonstrates and explicitly concludes that it is not possible without addition of ribonuclease inhibitor to samples during the freeze-thaw cycle to use gene-specific RT-PCR to detect, in RNA of a whole blood sample consisting of leukocytes which have not been fractionated into cell types the presence of RNA encoded by one of the most highly abundant, if not the most highly abundant, RNA species known, i.e. RNA encoded by beta-actin, where the sample been frozen and thawed in accordance with the pivotal cited teachings Sharma *et al.* This is clear from the recitation: "*As shown in Fig. 3, the production of... amplified beta-actin product was completely dependent on the inclusion of ... Ribonuclease Inhibitor in the reaction tube during the freeze-thaw cycle*" (Kephart, section titled "*RT-PCR with RNA isolated from human blood using a freeze-thaw protocol*", paragraph starting at bottom of page, and from Figure 3 itself which shows a complete absence of amplification product in lane 1 corresponding to the sample processed without added ribonuclease inhibitor. The prior art clearly teaches that beta-actin is so abundant as to possibly be the most abundant mRNA in all non-muscle cells, in accordance with the recitation: "*In all nonmuscle mammalian cells, beta-actin is one of the, if not the, most abundant mRNAs.*" in Gunning *et al.*, 1987. Proc Natl Acad Sci. 84: 4831–4835, page 4834; 1st paragraph of "Discussion" section. As is shown in the enclosed Abstract of Chirgwin (Chirgwin JM et al., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-9) guanidine thiocyanate and the reducing agent 2-mercaptoethanol are ribonuclease inhibitors, such being added to the blood sample after the freeze-thaw cycle according to the cited unsubstantiated teachings of Sharma *et al.*, whereas Kephart experimentally demonstrates that ribonuclease inhibitor must be present in the blood sample during the freeze-thaw cycle to enable subsequent

RT-PCR detection therein of even a maximally abundant gene-encoded RNA such as that encoded by the beta-actin gene.

Applicant further respectfully submits that the publication history of the inventors of Sharma *et al.* very strongly supports Applicant's position that at the time of the invention the ordinarily skilled artisan would not consider that the cited, yet unsubstantiated, teachings of Sharma *et al.* with regard to a method for isolation of mRNA from blood samples which have not been fractionated into cell types, as required by the claims, could be combined with the cited teachings of Ralph *et al.* so as to enable detection and differential quantitation of RNA encoded by a gene having disease-specific differential expression, as further required by the claims.

Specifically, the full publication history of the inventors of Sharma *et al.* does not appear to include any actual performance of a method demonstrating identification of a gene having disease-specific differential expression in RNA of a sample of blood which has not been fractionated into cell types according to the method described in the cited unsubstantiated teachings of Sharma *et al.* with regard to isolation of RNA at p. 35, section 5.1.1, namely utilizing freeze-thawing of blood samples in the absence of added ribonuclease inhibitor during the freeze-thaw cycle. In fact, the publication history of the inventors of Sharma *et al.* subsequent to the filing date of Sharma *et al.* reveals that the inventors of Sharma *et al.* had to resort to addition of ribonuclease inhibitors to blood samples which have not been fractionated into cell types prior to subjecting these samples to freeze-thawing for RNA isolation in order to allegedly achieve detection, in the isolated RNA, of RNA encoded by a gene as required by the claims. Specifically, during prosecution of US Pat Appl No. 09/429,003 ("003 application") having an apparently identical specification as that of Sharma *et al.*, i.e. teaching an apparently identical unsubstantiated RNA isolation method at section 5.1.1 as the one cited in Sharma *et al.*, the inventors of Sharma *et al.* filed Declarations under Rule 132 on 11/21/2002 and on 08/14/2003 to demonstrate that the teachings of the '003 application could be put into practice in accordance with the specification of the '003 application (Declaration filed 11/21/2002, page 1, section 1; and Declaration filed 08/14/2003, page 1, section 1), and hence further in accordance with the

cited teachings of Sharma *et al.* In particular, the Declarations describe multiple experiments in which ribonuclease inhibitor (EDTA or PAXgene tube RNA stabilizer) is always effectively added to whole blood samples immediately after collection, i.e. prior to subjecting the samples to freeze-thawing, in accordance with the recitations: "*whole blood was collected in tubes containing EDTA and stored immediately at –80°C until used for mRNA extraction.*—" (Declaration filed 11/21/2002, page 3, section 6; and Declaration filed 08/14/2003, page 4, section 7); and "*blood was either collected in tubes containing EDTA... or was collected in PAXgene tubes... before finally storing them at –80°C before use*" (Declaration filed 11/21/2002, page 5, section 13). The compound EDTA used in the experiments of the Declarations is a ribonuclease inhibitor as shown in the last paragraph of enclosed "Section 6.1.2 Ribonuclease degradation", excerpt from *Essential molecular biology.* By Terry Brown. Published by Oxford University Press, 1999. The PAXgene tubes used in the experiments of the Declaration contain a proprietary RNA stabilizer, i.e. ribonuclease inhibitor, as shown in the enclosed PreAnalytix-PAXgene Blood RNA System information sheet (2008). Thus, these Declarations by the inventors of Sharma *et al.* employ a method of RNA isolation via freeze-thawing of blood samples which is essentially in accordance with the teachings of Kephart as described above, as opposed to the cited teachings of Sharma *et al.* and of the '003 application itself at apparently identical section 5.1.1 of both of which.

Thus, the publication history of the inventors of Sharma *et al.* strongly suggests that the cited method taught at page 35, section 5.1.1 of Sharma *et al.* is not enabling, in accordance with the experimental data disclosed in the Kephart prior art reference described above, for isolation of RNA from a sample of blood which has not been fractionated into cell types, in which RNA encoded by a gene which is differentially expressed between subjects having a disease and control/healthy subjects can be detected by application of the cited teachings of Ralph *et al.*

Thus, in teaching that subjecting blood samples to a single freeze-thaw cycle in the absence of any stabilizing agent, such as a ribonuclease inhibitor, fails to enable detection of even RNA encoded by beta-actin, possibly the most abundant RNA in blood, Applicant

respectfully submits that Kephart *et al.*, which was cited in the Office Action dated 08/11/2006 as relevant prior art relative to the claimed invention, very convincingly leads the ordinarily skilled artisan at the time of the invention to consider that the pivotal yet unsubstantiated cited teaching of Sharma *et al.* at page 35, section 5.1.1, which teaches isolation of RNA of blood which has not been fractionated into cell types via freeze-thawing in the absence of ribonuclease inhibitor, could not be combined with the cited teachings of Ralph *et al.* so as to enable detection of a target RNA encoded by a gene, as contended by the Office Action, and as required by the claims. Thus, at the time of the invention, Kephart would strongly lead the ordinarily skilled artisan away from combining the cited teachings of Sharma *et al.* and Ralph *et al.*

As such, Applicant respectfully submits that the cited combination of Ralph *et al.* and Sharma *et al.* fails to render the claims obvious on the sole basis that the ordinarily skilled artisan at the time of the invention would not have considered the cited teachings of Sharma *et al.* to enable the cited combination of Sharma *et al.* in view of Ralph *et al.*, as described above, and hence would not have been motivated to combine the cited teachings of Sharma *et al.* and Ralph *et al.* so as to arrive at the claimed invention.

Applicant respectfully submits that there are additional compelling grounds to conclude that the claims are not rendered obvious by the cited teachings of Sharma *et al.* and of Ralph *et al.* Specifically, Applicant submits that it can be clearly shown in view of the particulars of the prior art, as described below, that at the time of the invention the ordinarily skilled artisan would not have had a reasonable expectation of success in achieving, as required by the claims, detection, in RNA of blood samples which have not been fractionated into cell types from control/healthy subjects, of RNA encoded by two or more genes which are differentially expressed between subjects having a disease and control/healthy subjects, for genes which have only been demonstrated to be differentially expressed in fractionated mononuclear cells between disease subjects and control/healthy subjects, as allegedly taught by the cited teachings of Ralph *et al.* Applicant respectfully submits that such lack of reasonable expectation of success is clearly evidenced by the strikingly consistent and counterintuitive fact pattern of the central paradigm

for RT-PCR analysis of disease biomarkers in blood at the time of the invention–namely, analysis of liver cancer via differential expression of the alpha-fetoprotein gene (*afp*) in blood. The centrality of this paradigm is clear from the recitation "*Detection of AFP and PSA mRNA by RT-PCR in peripheral blood has become one of the most useful molecular biomarkers (12,14-20) in cancer diagnosis.*" ("*Introduction*" section of enclosed Ishikawa *et al.*, 1998).

In particular, the prior art clearly teaches that *afp* RNA is detectable in fractionated mononuclear cells of healthy controls, in accordance with the recitation "*AFP mRNA was detected in peripheral blood from six out of seven healthy volunteers*" (see "*Detection of AFP and PSA mRNA by RT-PCR in Cancers and Normal Peripheral Blood*" section of enclosed Ishikawa *et al.*). [Note that Ishikawa *et al.* indeed use fractionated mononuclear cells in accordance with the recitation "*Whole blood was subjected to a Ficcoll-Conray gradient... and the nucleate cells were collected.*" ("*RNA Preparation and RT-PCR*" section of Ishikawa *et al.*). The term "Ficoll-Conray" has been misspelled "Ficcoll-Conray" and the otherwise unclear term "nucleate cells" clearly refers to mononuclear cells in this context since, as described below, subjecting whole blood to a Ficoll-Conray gradient (i.e. discontinuous gradient centrifugation on a Ficoll-Conray cushion) results in isolation of recoverable mononuclear cells at the interface and pelleting of a mixture of erythrocytes and granulocytes. The Ficoll-Conray method is essentially identical to the more commonly employed Ficoll-Hypaque discontinuous gradient centrifugation method for isolation of mononuclear cells (refer, for example, to enclosed Janeway Immunobiology 2006_Fig. A.23. Ficoll separation of PBMCs and granulocytes). The equivalence of Ficoll-Conray and Ficoll-Hypaque discontinuous density gradient centrifugation for isolation of mononuclear cells is described in the enclosed article of Onuma *et al.* (Onuma *et al.*, 1978. Ann Rech Vet 9:825-830 at page 827 under "*Cell Preparations*", according to the recitation "*Lymphocytes were separated from blood by a Ficoll-Hypaque gradient or Ficoll-Conray gradient as described by Boyum (1968).*" Isolated mononuclear cells are sometimes thusly referred to as "lymphocytes" in the art in accordance with the fact that mononuclear cells are composed of about 90% lymphocytes (refer, for example, to enclosed Janeway

Immunobiology 2006_Fig. A.23. "Ficoll separation of PBMCs and granulocytes", which demonstrates that mononuclear cells are composed of lymphocytes and "some" monocytes and the enclosed Alberts 2002_Blood cell proportions table, which demonstrates that mononuclear cells are composed of about 88% lymphocytes and about 12% monocytes). Confirmation that the Ficoll-Conray gradient centrifugation method used by Ishikawa *et al.* generates fractionated mononuclear cells is provided, for example, by the enclosed articles of: Hirokawa *et al.*, 2001. Bone Marrow Transplantation 27:1095-1100, at the "Materials and Methods Section", "Patients" subsection; Furukawa *et al.*, 1998. Annals Of Surgery 229:255-261, at the last paragraph of page 256; Iwata *et al.*, 1998. J Virol. 72:10044-10049, at the last paragraph of page 10044; Takahashi *et al.*, 2000, at the second-to-last paragraph of page 1410; and Yamamoto *et al.*, 1998. Clin Exp Immunol 114:94–101, at the second-to-last paragraph of page 95). Thus, Ishikawa *et al.* clearly teaches detection of *afp* mRNA in fractionated mononuclear cells of healthy subjects.]

In sharp and critical contrast, however, the prior art consistently and counterintuitively teaches, using highly sensitive RT-PCR methods using unfractionated blood cell RNA isolated by a variety of methods, and as performed by a variety of different artisans, that despite being detectable in fractionated mononuclear cells as demonstrated by Ishikawa *et al.*, *afp* RNA is undetectable in total blood cell RNA of unfractionated blood cells/leukocytes in healthy subjects, as required by the instant claims, in accordance with the following prior art references (attached hereto) constituting the aforementioned central art paradigm at the time of the invention:

Matsumura et al., 1994. Detection of alpha-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellular carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology. 20:1418. Similarly to Matsumura 1995, below, this reference analyzes *afp* RNA in whole nucleated blood cells (i.e. whole leukocytes) via nested RT-PCR. This reference explicitly teaches that *afp* RNA is not detectable in whole leukocytes of 26 healthy controls tested, in accordance with the recitation: "*alpha-fetoprotein mRNA was not demonstrated in 26 cases of normal healthy volunteers (0%)*" (Abstract; and 2nd paragraph of "RESULTS" section and Table 2). Isolation of RNA of unfractionated blood cells

is achieved by subjecting whole blood to erythrocyte lysis, recovering the remaining cells, and extracting the RNA thereof, as described at page 1419, second column.

Matsumura et al. 1995. **Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. Biochem Biophys Res Commun. 207:813.** This reference analyzes *afp* RNA in total RNA from whole nucleated blood cells (i.e. whole leukocytes) from healthy controls. This reference explicitly teaches, using the same RT-PCR primers as Matsumura *et al.*, 1994, above, that *afp* RNA is not detectable in whole leukocytes, in accordance with the recitation: "*Afp mRNA was not demonstrated in nuclear cell component of peripheral blood of healthy volunteers both by RT-PCR and even by nested PCR (Fig. 1)*" (p. 815, first paragraph of "RESULTS" section and Fig. 1). Isolation of RNA of unfractionated blood cells is described as subjecting whole blood to erythrocyte lysis, recovering the remaining cells, and extracting the RNA thereof, as described at page 814.

Funaki *et al.* 1995. **Highly-sensitive identification of alpha-fetoprotein mRNA in circulating peripheral blood of hepatocellular carcinoma patients. Life Sci. 57:1621.** This reference analyzes *afp* RNA in RNA of whole blood (p. 1622, last paragraph) via an extremely sensitive 3-step (double-nested) PCR method (Abstract). This reference explicitly teaches that *afp* mRNA is not detectable in whole blood of control subjects in accordance with the recitation: "*In contrast, no Afp mRNA transcript was found in blood samples from the 4 virus-infected and 1 healthy volunteer*" (p. 1627, last sentence). Isolation of RNA of unfractionated blood cells is described as subjecting whole blood to guanidinium-phenol-chloroform lysis, and isolating the RNA thereof, as described at the last paragraph of page 1622.

Lemoine *et al.* 1997. **Prospective evaluation of circulating hepatocytes by alpha-fetoprotein mRNA in humans during liver surgery. Ann Surg. 226:43.** This reference teaches isolation of whole nucleated blood cells (i.e. whole leukocytes) via erythrocyte lysis [p. 44 in the "*Alpha-Fetoprotein mRNA Assay*" section, and analysis of *afp* RNA from these cells via nested RT-PCR. This reference explicitly teaches that *afp* RNA was undetectable in whole leukocytes of all 28 non-disease controls (p. 48, col. 2, 1st paragraph). Isolation of RNA of unfractionated

blood cells is described as treating whole blood with tetradecyltrimethylammonium bromide (last paragraph of page 44) according to the method of MacFarlane and Dahle, 1993 (Lemoine *et al.*, reference #21, page 50).

Liu *et al.* 1998. The detection of circulating hepatocellular carcinoma cells in peripheral venous blood by reverse transcription-polymerase chain reaction and its clinical significance. Zhonghua Wai Ke Za Zhi. 36:608. This reference explicitly teaches that *afp* RNA is not detectable via RT-PCR (Abstract) in whole leukocytes of non-disease subjects in accordance with the recitation: "*there were no clinical control patients* [i.e. non-disease controls] *whose samples showed detectable Afp mRNA*" (Abstract). Isolation of RNA of unfractionated blood cells is described as "*alpha-fetoprotein (AFP) mRNA was amplified from total RNA extracted from whole blood*".

This extensive and paradigmatic body of experimental prior art therefore demonstrates that the cited teachings of Sharma *et al.* and of Ralph *et al.* do not render the claims obvious since it clearly teaches empirically and counterintuitively that there is not a reasonable expectation of success in achieving, as required by the claims, detection, in RNA of blood samples which have not been fractionated into cell types from control/healthy subjects, of RNA encoded by two or more genes which are differentially expressed between subjects having a disease and control/healthy subjects, for genes which have only been demonstrated to be differentially expressed in fractionated mononuclear cells between disease subjects and control/healthy subjects, as allegedly taught by the cited teachings of Ralph *et al.*

Applicant respectfully submits that the above-described prior art experimental data indicates that the Office Action has based the rejections on the intuitive but mistaken assumption that since a preparation of RNA of blood which has not been fractionated into cell types must contain RNA of mononuclear cells, then an RNA encoded by a gene having differential expression which is detectable in a preparation of RNA of fractionated mononuclear cells must therefore also be predictably detectable in the preparation of RNA of blood which has not been fractionated into cell types. The experimental evidence of the central prior art paradigm however

indicates that the ordinarily skilled artisan at the time of the invention would in fact consider it unlikely, as well as highly unpredictable, that any given RNA detected in a preparation of RNA of fractionated mononuclear cells will be necessarily detectable in a preparation of RNA of blood which has not been fractionated into cell types.

Applicant wishes to point out that it can be clearly shown as follows that simple dilution in a larger pool of total cell RNA, for example, clearly does not appear to explain the extensive and counterintuitive failure in the prior art to achieve detection of *afp* RNA in total cell RNA of unfractionated leukocytes of healthy controls, despite detectability in total cell RNA of fractionated mononuclear cells. Namely, mononuclear cells (lymphocytes + monocytes) typically account for a little over one-third of all cells included in unfractionated leukocytes (refer, for example, to enclosed "Alberts 2002_Blood cell proportions table"), and as such the RNA content of mononuclear cells, which are particularly transcriptionally active, will account for more than a little over one-third of the total RNA content of unfractionated leukocytes. As can be seen in the upper section of the upper panel of Figure 4 of Ishikawa *et al.*, *afp* RNA is clearly detectable, via RT-PCR amplification, at levels high enough that it is readily apparent that levels of *afp* RNA diluted to a little over one-third of those detected in Figure 4 of Ishikawa *et al.* would still be easily detectable.

Applicant wishes to further point out that it can be clearly shown as follows that differences in assay detection sensitivity does not appear to explain the extensive and counterintuitive failure in the prior art to achieve detection of *afp* RNA in total cell RNA of unfractionated leukocytes of healthy controls, despite detectability in total cell RNA of fractionated mononuclear cells. As can be seen in the "*RNA Preparation and RT-PCR*" section of Ishikawa *et al.*, RT-PCR amplification of *afp* RNA in 6 out of 7 controls is achieved via 50 cycles of standard, non-nested PCR amplification. In critical contrast, however, the various prior art studies systematically fail to achieve such detection despite employing significantly more sensitive, or similarly sensitive RT-PCR detection protocols than that employed by Ishikawa *et al.* on a far larger number of controls. Namely Matsumura *et al.*, 1994 fail to achieve detection of

afp RNA in total cell RNA of unfractionated leukocytes of 26 controls despite using nested RT-PCR with a total of 100 cycles of PCR amplification (Matsumura *et al.*, 1994, page 1420). Similarly, Matsumura *et al.*, 1995 fail to achieve detection of *afp* RNA in total cell RNA of unfractionated leukocytes of controls despite using nested RT-PCR with a total of 70 cycles of PCR amplification (Matsumura *et al.*, 1995, paragraph spanning pages 814-815). Lemoine *et al.* fail achieve detection of *afp* mRNA in total cell RNA of unfractionated leukocytes of any of 28 controls tested via standard RT-PCR. Liu *et al.* fail achieve detection of *afp* mRNA in total cell RNA of unfractionated leukocytes of any of 37 controls tested via RT-PCR. Funaki *et al.* fail to achieve detection of *afp* mRNA in total cell RNA of unfractionated leukocytes of controls despite employing hypersensitive double-nested RT-PCR with a total of 100 cycles of PCR amplification (Funaki *et al.*, pages 1624-1625).

Thus, neither dilution of *afp* RNA in total cell RNA of unfractionated leukocytes nor differences in assay detection sensitivity appears to account for the counterintuitive, unpredictable and empirically demonstrated failure in the central prior art paradigm to achieve detection of *afp* RNA in total cell RNA of unfractionated leukocytes, despite *afp* RNA being remarkably consistently and clearly detectable in RNA of fractionated mononuclear cells.

As such, Applicant respectfully submits that the cited combination of Ralph *et al.* and Sharma *et al.* fails to render the claims obvious on the sole basis that at the time of the invention the ordinarily skilled artisan would not have had a reasonable expectation of success in achieving, as required by the claims, detection, in RNA of blood samples which have not been fractionated into cell types from control/healthy subjects, of RNA encoded by two or more genes which are differentially expressed between subjects having a disease and control/healthy subjects, for genes which have only been demonstrated to be differentially expressed in fractionated mononuclear cells between disease subjects and control/healthy subjects, as allegedly taught by the cited teachings of Ralph *et al*

Applicant respectfully submits that there are yet further compelling grounds to conclude that the claims are not rendered obvious by the cited teachings of Sharma *et al.* and of Ralph *et*

al. Specifically, Applicant submits that it can be clearly shown that the cited combination of Sharma *et al.* and Ralph *et al.* does not teach all of the claim limitations.

To establish prima facie obviousness of a claimed invention, all the claim limitations must be taught or suggested by the prior art. In re Royka, 490 F.2d 981, 180 USPQ 580 (CCPA 1974).

The Office Action bases the rejections on the grounds that Ralph *et al.* teaches that responses secondary to disease states may be reflected in changing patterns of leukocyte mRNA levels that correlate with the presence of the disease state (Col. 5, lines 27-33).

The Office Action states at page 3 that Ralph *et al.* does not anticipate the claims, effectively by failing to teach the claimed RNA of a blood sample which has not been fractionated into cell types. Thus, in view of the Office Action, Ralph *et al.* is clearly limited to teaching RNA of fractionated mononuclear cells, which is in accordance with the extensive analyses with regard to the type of sample taught by Ralph *et al.* provided by Applicant previously during prosecution of the present application. Thus, Applicant respectfully submits that in combining the teachings of Ralph *et al.* and Sharma *et al.* so as to arrive at the claimed limitation of detecting the presence of a gene-specific RNA in RNA of a blood sample which has not been fractionated into cell types using a gene-specific oligonucleotide of predetermined sequence, such as via gene-specific RT-PCR, the Examiner has mistakenly assumed that such analysis consists of independent limitations which can be taken out of context so as to achieve substantially the same results. In particular, the Examiner has mistakenly assumed that the gene-specific RT-PCR component of the analysis of RNA of fractionated mononuclear cells via gene-specific RT-PCR allegedly taught by Ralph *et al.* can be used to analyze a blood sample which has not been fractionated into cell types, as claimed, so as to predictably achieve substantially the same result, i.e. so as to constitute the claimed limitation detecting the presence of a gene-specific RNA in RNA of a blood sample which has not been fractionated into cell types using a gene-specific oligonucleotide of predetermined sequence. Applicant submits, however, that it

can be clearly shown, as described above, that at the time of the invention the ordinarily skilled artisan would not consider analysis of RNA of a blood sample which has not been fractionated into cell types using the claimed oligonucleotide to correspond to analysis of RNA of fractionated mononuclear cells using the claimed oligonucleotide, i.e. would not consider these to be the same limitation. As shown above, at the time of the invention, the ordinarily skilled artisan would not expect to be able to be able to predictably detect, according to the claims, the presence of RNA encoded by a gene in RNA of a blood sample which has not been fractionated into cell types, using a gene-specific oligonucleotide of predetermined sequence, for a gene which had previously been detected only in fractionated mononuclear cells, as allegedly taught by Ralph *et al.* Thus, in failing to explicitly teach the combined limitation of analyzing a gene-specific RNA in a blood sample which has not been fractionated into cell types using an oligonucleotide of predetermined sequence specific for the gene, as claimed, Applicant submits that the combination of Ralph *et al.* and Sharma *et al.* fails to teach all of the limitations of the claims.

As such, Applicant respectfully submits that the cited combined teachings of Ralph *et al.* and Sharma *et al.* fail to render the claims obvious on the sole basis that these fail to teach all of the limitations of the claims.

As discussed below, predictability is required in maintaining a legal conclusion of obviousness under both KSR and the USPTO published guidelines. Applicant maintains that one of skill at the time of the invention could not have reliably predicted that whole blood contained a single biomarker, never mind two or more biomarkers, for any disease. Sharma et al. does not exemplify a single blood RNA biomarker for any disease. Ralph et al. teaches 2 blood RNA biomarkers differentially expressed in cancer in fractionated cells. Given that none of the cited references exemplifies even a single RNA marker which is differentially expressed with respect to disease in blood samples which have not been fractionated into cell types, the cited teachings do not provide a substantive basis for reliably predicting that there is even a single RNA biomarkers in whole blood useful for diagnosing disease. The Office action states that "the fact

that Ralph et al. and Sharma et al. are considered skilled in their fields and that they set forth their teachings very clearly enjoys a presumption that they are valid". Applicant with respect, disagrees. Applicant contends that broad hypotheses without any supporting data is not a basis for predictability under KSR and the USPTO published guidelines, especially in an art in its infancy (note not a single RNA biomarker for disease in whole blood has been exemplified in any of the cited references) in a field, biotechnology, that the MPEP characterizes as unpredictable, no matter the skill level of the author. The Office Action states that "there is no evidence on the record to suggest that their teachings are not valid" in referring to the teachings of Ralph et al. and Sharma et al. However, the office has the burden of making a prima facie case of obviousness at the time of the invention, which Applicant respectfully contends can not be supported on the evidence provided by Ralph et al. and Sharma et al.

In light of the above remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejections.

Claims 17, 20, 23, 28-29, 33-34, 41, 43, 49 and 59-63 are rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma et al. in view of Lockhart et al.

Applicant submits that the cited combination of teachings fails to render the claims obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that Sharma *et al.* teaches a method for isolation of mRNA from blood samples which have not been fractionated into cell types which would not be considered reasonably enabling by the ordinarily skilled artisan such that combination of this teaching with the cited teachings of Lockhart *et al.* could be used to achieve detection of RNA encoded by a gene in RNA of a blood sample which has not been fractionated into cell types, as required by the claims. Additionally, since RT-PCR, as allegedly taught by Ralph *et al.*, is a significantly more sensitive method than the microarray method taught by Lockhart *et al.*, and since, as explained above, the ordinarily skilled artisan would not consider the teachings of Sharma *et al.* to enable RNA detection according to the claims, the ordinarily skilled artisan would *a fortiori*

not consider the cited combined teachings of Sharma *et al.* and Lockhart *et al.* to enable the claims.

Applicant further submits that the cited combination of teachings fails to render the claims obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that the cited combined teachings of Ralph *et al.* and Lockhart *et al.* fail to teach all of the limitations of the claims.

In light of the above remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejections.

Graham v. John Deere Co., 338 U.S. 1, 148 USPQ 459 (1966), recently reaffirmed by *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727, 82 USPQ2d 1385 (2007), provides the analytical framework for determining obviousness. Under *Graham*, obviousness is a question of law based on underlying factual inquires that address (1) the scope and content of the prior art, (2) the differences between the claimed invention and the prior art, and (3) the level of ordinary skill in the pertinent art. Evidence of secondary factors (e.g., commercial success, long-felt but unmet need, and unexpected results) are also given weight in the analysis. Moreover, to establish a prima facie obviousness rejection of a claimed invention, all the claim limitations must be taught or suggested by the prior art. *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974). Predictability is required in maintaining a legal conclusion of obviousness under both KSR and the USPTO published guidelines. Applicant respectfully traverses.

The thrust of the rebuttals presented below is that the claimed invention is not predictably arrived at in light of the following factors when perceived by one of skill at the time of the invention: (1) the scope and content of the prior art, (2) the differences between the claimed invention and the prior art, and (3) the level of ordinary skill in the pertinent art.

Without evidence that one of skill in the art could have predictably arrived at the claimed invention based on the teachings of the cited references, a prima facie case of obviousness has not been achieved.

In noting that Wei et al. teaches that expression of IL-6 mRNA is known to increase in the blood of diabetic patients, the office action contends that it would have been obvious to have applied the methods of Sharma et al. in view of Ralph et al. to the disease heart failure in order to identify additional markers in the blood that would be useful for detecting and understanding this disease" using the motivation that "one would have reasonably expected to identify numerous additional markers in RNA extracted from whole blood...". However, Applicant respectfully traverses the idea that one of skill at the time of the invention could have reliably predicted that whole blood contained a single biomarker, never mind two or more biomarkers, for diabetes. Sharma et al. does not exemplify a single blood biomarker for any disease, including diabetes. Ralph et al. teaches 2 blood biomarkers differentially expressed in cancer in fractionated cells. Wei et al. teaches that IL-6 mRNA is differentially expressed in fractionated blood cells from diabetic patients as opposed to whole blood (e.g., in blood samples which have not been fractionated into cell types) as required by the instant claim. Given that none of the cited references exemplifies even a single RNA marker which is differentially expressed with respect to diabetes in blood samples which have not been fractionated into cell types, the cited teachings do not provide a substantive basis for reliably predicting that there is even a single RNA biomarkers in whole blood useful for diagnosing diabetes. Lockhart's teaching of methods of using a microarray do not provide any predictability that RNA biomarkers for diabetes are present in whole blood.

In light of the above remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejections.

Claim 59 is rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* in view of either Ralph *et al.* as applied to claims 17 and 19 and further in view of Wei *et al.*

Applicant submits that the cited combination of teachings fails to render the claim obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that Sharma *et al.* teaches a method for isolation of mRNA from blood

samples which have not been fractionated into cell types which would not be considered reasonably enabling by the ordinarily skilled artisan such that combination of this teaching with the cited teachings of Ralph *et al.* and Wei *et al.* could be used to achieve detection of RNA encoded by a gene in RNA of a blood sample which has not been fractionated into cell types, as required by the claims.

Applicant additionally submits that the cited combination of teachings fails to render the claim obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that at the time of the invention the ordinarily skilled artisan would not have had a reasonable expectation of success in achieving, as required by the claims, detection, in RNA of blood samples which have not been fractionated into cell types from control/healthy subjects, of RNA encoded by two or more genes which are differentially expressed between subjects having a disease and control/healthy subjects, for genes which have only been demonstrated to be differentially expressed in fractionated mononuclear cells between disease subjects and control/healthy subjects, as allegedly taught by the cited teachings of Ralph *et al.* and Wei *et al.*

Applicant further submits that the cited combination of teachings fails to render the claims obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that the cited combined teachings of Sharma *et al.*, Ralph *et al.* and Wei *et al.* fail to teach all of the limitations of the claims.

Without evidence that one of skill in the art could have predictably arrived at the claimed invention based on the teachings of the cited references, a prima facie case of obviousness has not been achieved, as discussed above. In noting that Wei et al. teaches that expression of IL-6 mRNA is known to increase in the blood of diabetic patients, the office action contends that it would have been obvious to have applied the methods of Sharma et al. in view of Ralph et al. to the disease heart failure in order to identify additional markers in the blood that would be useful for detecting and understanding this disease" using the motivation that "one would have reasonably expected to identify numerous additional markers in RNA extracted from whole

blood...". However, Applicant respectfully traverses the idea that one of skill at the time of the invention could have reliably predicted that whole blood contained a single biomarker, never mind two or more biomarkers, for diabetes. Sharma et al. does not exemplify a single blood biomarker for any disease, including diabetes. Ralph et al. teaches 2 blood biomarkers differentially expressed in cancer. Wei et al. teaches that IL-6 mRNA is differentially expressed in fractionated blood cells from diabetic patients as opposed to whole blood (e.g., in blood samples which have not been fractionated into cell types) as required by the instant claim. Given that none of the cited references exemplifies even a single RNA marker which is differentially expressed with respect to diabetes in blood samples which have not been fractionated into cell types, the cited teachings do not provide a substantive basis for reliably predicting that there is even a single RNA biomarkers in whole blood useful for diagnosing diabetes.

In light of the above remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejections.

Claim 60 is rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* (WO 98/49342) in view of either Ralph *et al.* (US 6109857 and WO 98/24953) as applied to claims 17 and 19, and further in view of Kasuga *et al.*

Applicant submits that the cited combination of teachings fails to render the claim obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that Sharma *et al.* teaches a method for isolation of mRNA from blood samples which have not been fractionated into cell types which would not be considered reasonably enabling by the ordinarily skilled artisan such that combination of this teaching with the cited teachings of Ralph *et al.* and Kasuga *et al.* could be used to achieve detection of RNA encoded by a gene in RNA of a blood sample which has not been fractionated into cell types, as required by the claims.

Applicant additionally submits that the cited combination of teachings fails to render the claim obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that at the time of the invention the ordinarily skilled artisan would not have had a reasonable expectation of success in achieving, as required by the claims, detection, in RNA of blood samples which have not been fractionated into cell types from control/healthy subjects, of RNA encoded by two or more genes which are differentially expressed between subjects having a disease and control/healthy subjects, for genes which have only been demonstrated to be differentially expressed in fractionated mononuclear cells between disease subjects and control/healthy subjects, as allegedly taught by the cited teachings of Ralph *et al.* and Kasuga *et al.*

Applicant further submits that the cited combination of teachings fails to render the claims obvious on sole grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections that the cited combined teachings of Sharma *et al.*, Ralph *et al.* and Kasuga *et al.* fail to teach all of the limitations of the claims.

Without evidence that one of skill in the art could have predictably arrived at the claimed invention based on the teachings of the cited references, a prima facie case of obviousness has not been achieved, as discussed above. In noting that Kasuga et al. teaches that expression of monocyte chemotactic and activating factor mRNA is known to increase in the blood of acute heart failure patients, the office action contends that "it would have been obvious to have applied the methods of Sharma et al. in view of Ralph et al. to the disease heart failure in order to identify additional markers in the blood that would be useful for detecting and understanding this disease" using the motivation that "one would have reasonably expected to identify numerous additional markers in RNA extracted from whole blood...". However, Applicant respectfully traverses the idea that one of skill at the time of the invention could have reliably predicted that whole blood contained additional biomarkers for heart disease. Sharma et al. does not exemplify a single blood biomarker for any disease, including heart disease. Ralph et al. teaches two blood biomarkers differentially expressed in cancer in fractionated cells. Kasuga et al. does

not teach whether the increase in expression of monocyte chemotactic and activating factor mRNA occurs in whole blood (e.g., in blood samples which have not been fractionated into cell types) as required by the instant claim. Given that none of the cited references exemplifies even a single RNA biomarker which is differentially expressed with respect to heart disease in blood samples which have not been fractionated into cell types, the cited teachings do not provide a substantive basis for reliably predicting that there is even a single RNA biomarker, never mind two or more RNA biomarkers, in whole blood useful for diagnosing heart failure.

In light of the above remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejections.

Claim 59 is rejected under 35 U.S.C. 103(a) as being unpatentable over Sharma *et al.* (WO 98/49342) in view of Lockhart *et al.* as applied to claims 17 and 19, and further in view of Kasuga *et al.*

Applicant submits that the cited combination of teachings fails to render the claim obvious on grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections, that Sharma *et al.* teaches a method for isolation of mRNA from blood samples which have not been fractionated into cell types which would not be considered reasonably enabling by the ordinarily skilled artisan such that combination of this teaching with the cited teachings of Lockhart *et al.* and Kasuga *et al.* could be used to achieve detection of RNA encoded by a gene in RNA of a blood sample which has not been fractionated into cell types, as required by the claims. Additionally, since RT-PCR, as allegedly taught by Ralph *et al.*, is a significantly more sensitive method than the microarray method taught by Lockhart *et al.* and since, as explained above, the ordinarily skilled artisan would not consider the teachings of Sharma *et al.* to enable RNA detection according to the claims, the ordinarily skilled artisan would *a fortiori* not consider the cited combined teachings of Sharma *et al.*, Lockhart *et al.* and Kasuga *et al.* to enable the claims.

Applicant further submits that the cited combination of teachings fails to render the claims obvious on grounds as described above in Applicant's response to the Sharma *et al.* in view of Ralph *et al.* rejections, that the cited combined teachings of Ralph *et al.*, Lockhart *et al.* and Kasuga *et al.* fail to teach all of the limitations of the claims.

Without evidence that one of skill in the art could have predictably arrived at the claimed invention based on the teachings of the cited references, a prima facie case of obviousness has not been achieved.

. In noting that Kasuga et al. teaches that expression of monocyte chemotactic and activating factor mRNA is known to increase in the blood of acute heart failure patients, the office action contends that "it would have been obvious to have applied the methods of Sharma et al. in view of Ralph et al. to the disease heart failure in order to identify additional markers in the blood that would be useful for detecting and understanding this disease" using the motivation that "one would have reasonably expected to identify numerous additional markers in RNA extracted from whole blood...".

However, Applicant respectfully traverses the idea that one of skill at the time of the invention could have reliably predicted that whole blood contained additional biomarkers for heart disease. Sharma et al. does not exemplify a single blood biomarker for any disease, including heart disease. Ralph et al. teaches two blood biomarkers differentially expressed in cancer in fractionated cells. Kasuga et al. does not teach whether the increase in expression of monocyte chemotactic and activating factor mRNA occurs in whole blood (e.g., in blood samples which have not been fractionated into cell types) as required by the instant claim. Given that none of the cited references exemplifies even a single RNA biomarker which is differentially expressed with respect to heart disease in blood samples which have not been fractionated into cell types, the cited teachings do not provide a substantive basis for reliably predicting that there is even a single RNA biomarker, never mind two or more RNA biomarkers, in whole blood useful for diagnosing heart failure. Lockhart's teaching of methods of using a

microarray do not provide any predictability that RNA biomarkers for diabetes are present in whole blood.

In light of the above remarks, Applicant very respectfully requests reconsideration and withdrawal of the instant rejections.

Double Patenting

The office action indicates that the previously set forth rejections of obviousness type double patenting are maintained and applied to claims 62-63. Applicant respectfully traverses the rejection, but will consider filing a terminal disclaimer upon allowance.

Conclusion

Applicant submits that all claims are allowable as written and respectfully request early favorable action by the Examiner. If the Examiner believes that a telephone conversation with Applicant's attorney/agent would expedite prosecution of this application, the Examiner is cordially invited to call the undersigned attorney/agent of record.

Respectfully submitted,

Dated: March 17, 2009

Respectfully submitted,

Amy De Clerq 54642 for

Kathleen Williams
Registration No.: 34,380
Customer No. 21874
EDWARDS ANGELL PALMER & DODGE LLP
P.O. Box 55874
Boston, Massachusetts 02205
(617) 239-0294
Attorneys/Agents For Applicant

Encl.:

Alberts 2002_Blood cell proportions table

Chirgwin JM *et al.*, 1979. Biochemistry. 18(24):5294-9 (Abstract);

Funaki et al., 1995. Life Sciences 57:1621-1631;

Furukawa et al., 1998. Annals Of Surgery 229:255-2616;

Hirokawa *et al.*, 2001. Bone Marrow Transplantation 27:1095-1100;

Ishikawa *et al.*, 1998. Japanese Journal of Clinical Oncology 28: 723-728;

Iwata et al., 1998. J Virol. 72:10044-10049:

Janeway Immunobiology, 2006,_Fig. A.23. Ficoll separation of PBMCs and granulocytes;

Lemoine et al., 1997. Annals of Surgery 226:43-50;

Liu et al., 1998.Zhonghua Wai Ke Za Zhi 36:608-10 (Abstract).

Matsumura et al., 1994. Hepatology 20:1418-1425;

Matsumura et al., 1995. Biochem Biophys Res Comm 207:813-818;

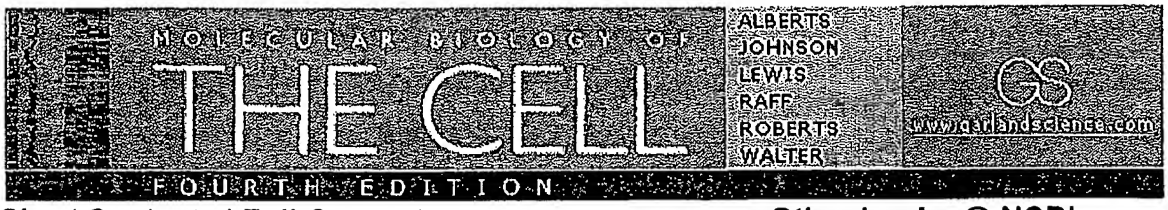
Onuma *et al.*, 1978. Ann Rech Vet 9:825-830;

PreAnalytix-PAXgene Blood RNA System information sheet (2008);

"Section 6.1.2 Ribonuclease degradation", excerpt from *Essential molecular biology.* By Terry Brown. Published by Oxford University Press, 1999

Takahashi et al., 2000, at the second-to-last paragraph of page 1410; and

Yamamoto et al., 1998. Clin Exp Immunol 114:94–101.



Short Contents | Full Contents Other books @ NCBI

Navigation

About this book

V. Cells in Their Social Context

22. Histology: The Lives and Deaths of Cells in Tissues

Epidermis and Its Renewal by Stem Cells

Sensory Epithelia

The Airways and the Gut

Blood Vessels and Endothelial Cells

Renewal by Multipotent Stem Cells: Blood Cell Formation

Genesis, Modulation, and Regeneration of Skeletal Muscle

Fibroblasts and Their Transformations: The Connective-Tissue Cell Family

Stem-Cell Engineering

References

Search

Molecular Biology of the Cell ➔ **V. Cells in Their Social Context** ➔ 22. Histology: The Lives and Deaths of Cells in Tissues ➔ Renewal by Multipotent Stem Cells: Blood Cell Formation

Table 22-1. Blood Cells

TYPE OF CELL	MAIN FUNCTIONS	TYPICAL CONCENTRATION IN HUMAN BLOOD (CELLS/LITER)
Red blood cells (erythrocytes)	transport O_2 and CO_2	5×10^{12}
White blood cells (leucocytes)		
Granulocytes		
Neutrophils (polymorphonuclear leucocytes)	phagocytose and destroy invading bacteria	5×10^9
Eosinophils	destroy larger parasites and modulate allergic inflammatory responses	2×10^8
Basophils	release histamine (and in some species serotonin) in certain immune reactions	4×10^7
Monocytes	become tissue macrophages, which phagocytose and digest invading microorganisms and foreign bodies as well as damaged senescent cells	4×10^8
Lymphocytes		
B cells	make antibodies	2×10^9

T cells	kill virus-infected cells and regulate activities of other leucocytes	1×10^9
Natural killer (NK) cells	kill virus-infected cells and some tumor cells	1×10^8
Platelets (cell fragments arising from *megakaryocytes* in bone marrow)	initiate blood clotting	3×10^{11}

Humans contain about 5 liters of blood, accounting for 7% of body weight. Red blood cells constitute about 45% of this volume and white blood cells about 1%, the rest being the liquid blood plasma.

A2

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ., 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry. 18(24):5294-9.

Intact ribonucleic acid (RNA) has been prepared from tissues rich in ribonuclease such as the rat pancreas by efficient homogenization in a 4 M solution of the potent protein denaturant guanidinium thiocyanate plus 0.1 M 2-mercaptoethanol to break protein disulfide bonds. The RNA was isolated free of protein by ethanol precipitation or by sedimentation through cesium chloride. Rat pancreas RNA obtained by these means has been used as a source for the purification of alpha-amylase messenger ribonucleic acid.

PMID: 518835 [PubMed - indexed for MEDLINE]

A3

Pergamon

Life Sciences, Vol. 57, No. 17, pp. 1621–1631, 1995

0024-3205/95 $9.50 + .00

0024-3205(95)02138-8

HIGHLY-SENSITIVE IDENTIFICATION OF α -FETOPROTEIN mRNA IN CIRCULATING PERIPHERAL BLOOD OF HEPATOCELLULAR CARCINOMA PATIENTS

Naomi Funaki, Junji Tanaka, Shin-ichi Seto, Takayuki Kasamatsu, Toshimi Kaido and Masayuki Imamura.

First Department of Surgery, Faculty of Medicine,
Kyoto University,
54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606, Japan

(Received in final form August 21, 1995)

Summary

In order to capture hepatocellular carcinoma (HCC) cells in circulating peripheral blood, we made analysis to see if α -fetoprotein (AFP) mRNA exists in the peripheral blood obtained from patients with HCC and also, as a control, from hepatitis-viral-marker-positive patients without HCC and a healthy volunteer. As the number of HCC cells in peripheral blood and the quantity of AFP mRNA are expected to be very small, the analysis was performed by the reverse transcription followed by an original three-step polymerase chain reaction. By this highly-sensitive method, 5 of 7 HCC patients were positive for AFP mRNA. These 5 positive patients consisted of three with clinically apparent recurrence, one preoperative patient with tumor thrombus in the portal vein and one recurrence-free patient who developed clinically detectable recurrence three months after this analysis. Neither 4 patients with positive viral markers nor a healthy volunteer was positive. The results suggest that detection of AFP mRNA from HCC patients' peripheral blood by our highly-sensitive RT-PCR may be a practical and powerful tool to diagnose the preoperative spreading of HCC and to monitor its recurrence.

Key Words: AFP mRNA, three-step RT-PCR, hepatocellular carcinoma, hematogenous disseminations

---

Address correspondence to : Naomi Funaki, M.D.
First Department of Surgery, Faculty of Medicine, Kyoto University
54 Shogoinkawahara-cho, Sakyo-ku, Kyoto 606, Japan

It is ideal to perform curative liver resection for all HCC patients. But in many cases the poor hepatic functional reserve due to co-existing chronic hepatitis or liver cirrhosis limits the range of safe resection. Moreover, even if we could manage to perform curative resection, not a few patients develop recurrence. In addition, in recent years more conservative therapeutic choices such as transcatheter arterial embolization (TAE) and percutaneous ethanol infusion therapy (PEI) have been practiced. Therefore, if we could know more precisely before operation whether a patient's HCC is localized or has unhappily hematogenously disseminated, we would be able to choose more appropriate therapy for individual patients taking both the stage of HCC and hepatic functional reserve into consideration. Furthermore, the capture of circulating HCC cell(s) from a post-operative patient will enable us to predict his high possibility of recurrence, and so will tell us the timing of imaging diagnosis, or even an intensive preventive chemotherapy. By these reasons, we tried to detect HCC cells circulating in the peripheral blood as a form of AFP mRNA. AFP is a well-known oncofetal protein and its serum level is used in screening HCC (1). But secreted AFP protein does not mean the presence of "cell" in the circulation (2). In contrast, the presence of AFP mRNA in the circulation, that is, the existence of AFP-producing cell(s) in blood is thought to be highly suggestive of the existence of circulating HCC. The present study describes our successful capture of AFP mRNA from peripheral blood of pre- and post-operative HCC patients with high efficiency.

Patients and Methods

Patients All patients' profiles are listed on Table I .

RNA extraction and cDNA synthesis RNA was extracted from peripheral blood according to the acid guanidinium-phenol-chloroform (AGPC) method (3) with a slight modification. In short, 5ml of heparinized whole blood was mixed well with 5ml of a guanidine isothiocyanate-enriched solution D [5M-guanidine isothiocyanate/ 25mM-sodium citrate(pH7.0)/0.5% Sarcosyl/100mM- β -mercaptoethanol]. We increased the quantity of guanidine isothiocyanate for protection of RNA in the whole blood according to the report of Gillespie et al (4). Two ml of this sample mixure, which

TABLE I

Patient number	Sex (M/F)	Age (yr)	HCC	clinical findings	viral*1 marker	serum AFP (ng/ml)	step of detection
1.	M	65	+	R(lung)*2 LC *3	HBsAb, HCVAb	4325	second
2.	F	60	+	R(liver)	HBsAb	83	second
3.	M	66	+	R(liver) LC	HBsAb, HCVAb	18	second
4.	F	45	+	pre*4 LC	HBsAg	210	third
5.	M	56	+	R(-) LC	HBsAb	4	third
6.	M	74	+	pre LC	HCVAb	67213	-
7.	M	68	+	R(-) LC	negative	4	-
8.	M	35	-	NC*5	negative	<4	-
9.	F	54	-	CH*6	HBsAb, HCVAb	12	-
10.	F	80	-	LC	HCVAb	<4	-
11.	M	64	-	none*7	HCVAb	<4	-
12.	F	90	-	none	HBsAg	<4	-

*1 Positive marker(s) listed

*2 R indicates recurrence with the site in parentheses

*3 LC indicates liver cirrhosis

*4 'pre' indicates preoperative case

*5 NC indicates normal control

*6 CH indicates chronic hepatitis

*7 'none' in these patients means that there is no liver dysfunction at present

corresponds to 1ml of total blood, was further processed by the ordinary AGPC method. Extracted RNA was solubilized in diethyl pyrrocarbonate (DEPC)-treated water (4) and was reverse-transcribed in a 50 μ l mixure consisted of 10μ l of 5× buffer (Gibco BRL), 2mM dNTP (Wako Pure Chemical Industries,LTD,Japan), 10mM DTT (Gibco), 0.25μ g random hexamer (Pharmacia), 5 μ g bovine serum albumin (BSA)(Gibco) and 200U M-MLV reverse transcriptase (Gibco,Cat.No. 28025-013). The reverse transcription was performed at 37 ℃ for one hour.

Preparation of positive control template a high-AFP-producing human HCC cell line, HuH7 (Gift from JCRB) (5) was cultivated in Dulbecco's modified eagle's medium supplemented with 10% fetal

bovine serum. mRNA was extracted from HuH7 cells using a Quick Prep® mRNA purification kit (Pharmacia) and then reverse transcription was performed using 1 μg of mRNA according to the above-described method.

PCR primers The primers for AFP gene (6,7) detection were sense primer 1 (5'-ATTCAGACTGCTGCAGCCAA-3') and sense primer 2 (5'-GTTCCAGAACCTGTCACAAG-3') both within exon 4, sense primer 3 (5'-TGGGACCCGAACTTTCCAAG-3') within exon 6, and common anti-sense primer 4 (5'-GTGCTCATGTACATGGGCCA-3') within exon 7.

PCR protocol First step-PCR was performed using primers 1 and 4 which amplify a 476-bp fragment. The 50 μl of individual PCR mixure was composed of one half of the reverse-transcribed sample (25μl) and 5μl of 10 × PCR buffer (Perkin-Elmer-Cetus, Norwalk,CT), 50pmole of each primer in 1μl of TE(pH8.0), 1 μg of BSA, 0.2mM dNTP and DEPC-treated water. The reaction mixure was overlaid with mineral oil, heat-denatured at 93 ℃ for 3minutes and then cooled to 80℃ for addition of 2.5 units of Taq polimerase (AmpliTaq®, Perkin-Elmer-Cetus). Each cycle of amplification consisted of 1-second denature at 94℃, followed by 20-second annealing (63℃) and 30-second extension (72 ℃). After 35 cycles, the final product was extended for 10 more minutes. One-fiftieth of the first PCR product was used as the template for the second-step PCR. The second set of primers consisted of primers 2 and 4 which amplify a 384-bp fragment. These primers were added by 50pmole individually. Another constituent of the second PCR mixture was the same as the first PCR mixure except for the addition of 24μl of DEPC-treated water. The amplification program for the second PCR was the same as the first PCR. One-fiftieth of the second PCR product was used as the template for the third PCR. The third primers were 3 and 4, amplifying a 115-bp fragment. Except for the difference of primers, the third PCR mixture was the same as the second PCR mixure. The annealing step for the third PCR was performed at 65℃ for 30 seconds and 30 cycles of amplification was practiced. The rest of the amplification program for the third PCR was the same as the previous PCR. 10μl of the PCR product was subjected to electrophoresis unless noted on 2.5% agarose gels (Agarose NA,Pharmacia Biotech,Sweden) containing 20ng/ml ethidium bromide.

To test the reliability of RNA extraction, a 319-bp β-actin cDNA fragment was amplified using the 50-pmole-each primer pair reported by Fuqua et al (8). The PCR template was the residual

one half of sample cDNA. The constituent of the PCR mixture was the same as the mixture for AFP mRNA except for the difference of primers. Our PCR protocol for the detection of β-actin cDNA fragment consisted of 3-minute heat-denature at 93℃, Taq polimerase addition at 80 ℃, and then 1-second heat denature (94 ℃), followed by 30-second annealing (55℃) and 30-second extension (72 ℃) steps. After 35 cycles, the final product was extended for 10 more minutes. When the amplified band was faint, one-fiftieth of the PCR product (corresponding 1 μl) was amplified in a new PCR mixure for 30 cycles using the same primer set under the same program.

The positive control for AFP mRNA detection was the PCR performed with 0.1μg of HuH7 mRNA. The negative control for the first step was the simultaneously-performed PCR without any template. The negative control for the second- and third-step was the PCR performed with one-fiftieth (corresponding to 1 μl) of the negative control in the previous PCR.

Results

PCR amplification on HuH7 As is observed in Figure 1, the first, second or third PCR products were visible as a clear single band individually and non-specific additional bands were not observed either on positive lanes or negative control lanes.

Detection of AFP mRNA in serially diluted HuH7 cDNA To determine the sensitivity of our assay, 0.1 μg of HuH7 cDNA was serially diluted and used as the PCR template. The first PCR made a 10^{-2}-diluted sample visible (data not shown). The second PCR visualized a 10^{-5}-diluted sample (Figure 2A), and the third PCR increased the visible range up tp 10^{-8} dilution (Figure 2B).

Detection of AFP mRNA in small quantity of HuH7 cells mixed with 1ml of AFP mRNA-negative normal healthy volunteer's blood 10^4, 10^3, 10^2, 10, 5 or 1 HuH7 cells were mixed with 1ml of healthy volunteer's blood, which had been pre-checked as negative for AFPmRNA using our system. Total RNA extraction, cDNA synthesis and PCR amplification were performed just as was done on patients' blood. Under our system even one HuH7 cell-containing blood sample was positive for AFP mRNA and the blood sample without HuH7 cells was negative (Figure 3).

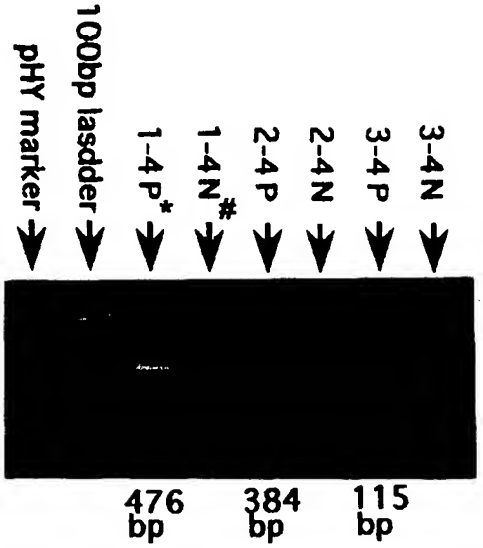


FIG.1

PCR amplification of HuH7 cDNA forms a clear single band. *P is PCR product with template and *N is that without template. Numbers 1,2,3 and 4 represent the name of primer. pHY marker indicates 4870,2016,1360,1107,926,658,489, 267 and 80bp fragments. The intermediate bright band of 100 bp-ladder indicates 600bp DNA.

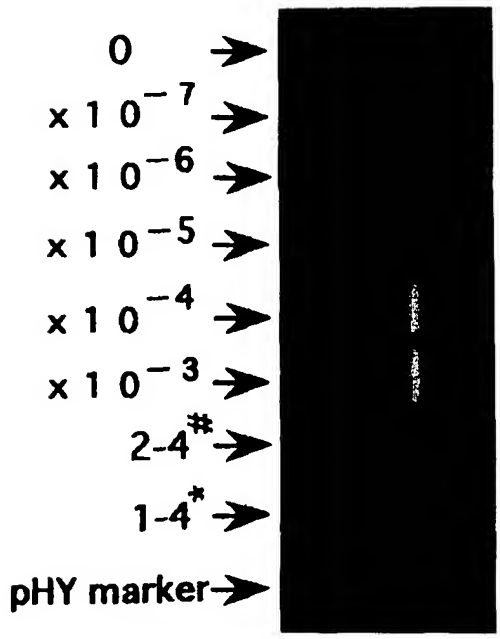


FIG.2A

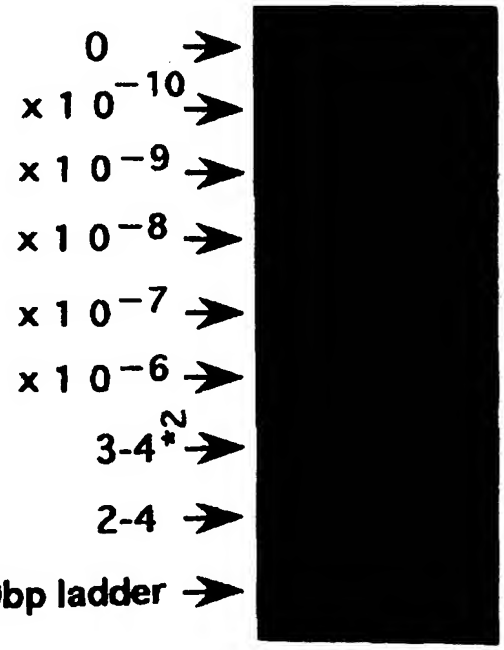


FIG.2B

PCR using serially diluted HuH7-derived cDNA to test the sensitivity. Fig.2A represents the second PCR product and Fig.2B the third PCR product. 1-4*, 2-4# and 3-4*² are the positive control lane with indicated primer pair.

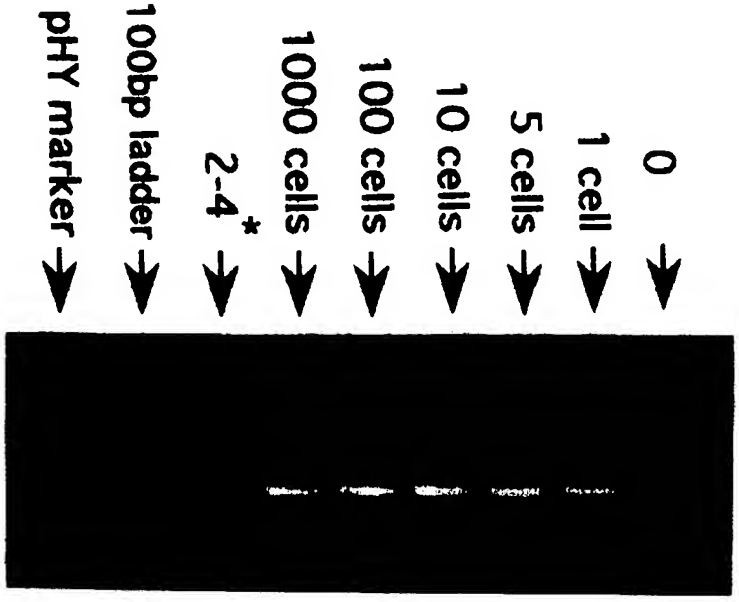


FIG.3
Even one HuH7 cell contaminated in 1ml of healthy volunteer's blood was detectable at the second PCR. 2-4* indicate the positive control product with the second primer pair. Of the total 50μ l of PCR product, we used 5 μ l for the 1000-cell and 100-cell samples, and 15μ l for the 10-cell sample, and 30μ l for the 5-cell and one-cell samples, individually.

Detection of AFP mRNA in patients' samples Among 7 HCC patients, 5 were positive for AFP mRNA (71%)(Figure 4). Of these 5 patients, No.1, 2 and 3 patients were already positive on the second PCR. No.1 patient had lung metastasis and No.2 and No.3 patients had multiple intrahepatic recurrence (Table 1). No.6 preoperative patient, whose serum AFP protein level was as high as 67213 ng/ml was negative for AFP mRNA. When we performed the PCR, two more patients (No.4 and 5) were found to be positive. No.4 preoperativepatient whose portal venogram showed tumor thrombosis was positivefor AFP mRNA. No.5 follow-up patient in whom no recurrence was detected at the point of blood sampling was positive for AFP mRNA, and within three months showed an evident recurrence on the echograms. No.7 follow-up patient without any clinical recurrence and negative for AFP mRNA showed no recurrence even after three months. In contrast, no AFP mRNA transcript was found in blood samples from the 4 virus-infected patients and 1 healthy volunteer (Figure 4).

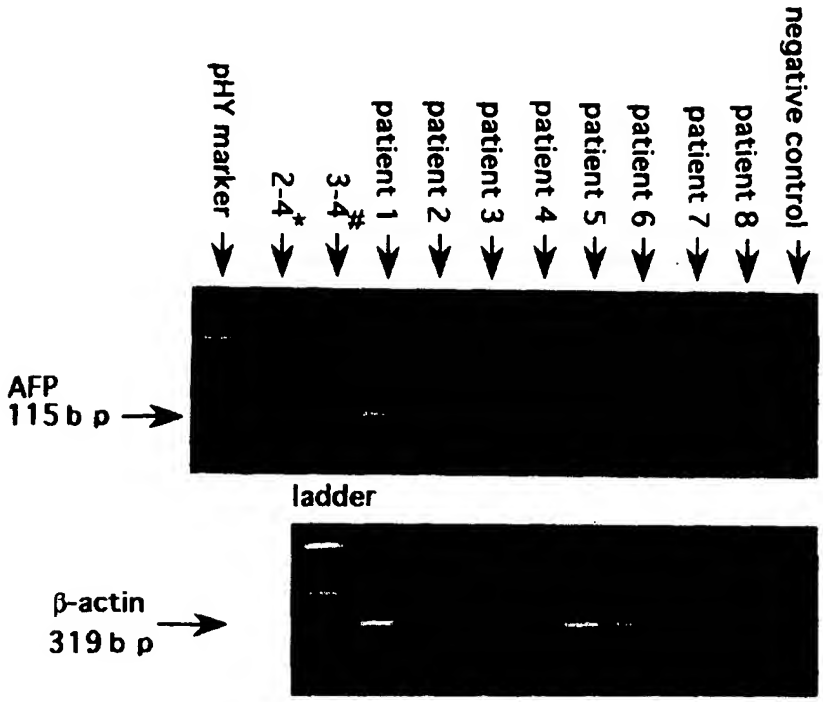


FIG. 4A

The third step PCR amplification of HCC patients' sample (1-7) and a healthy volunteer's sample (8). 2-4* and 3-4# indicate the positive control for the second and third PCR, individually.

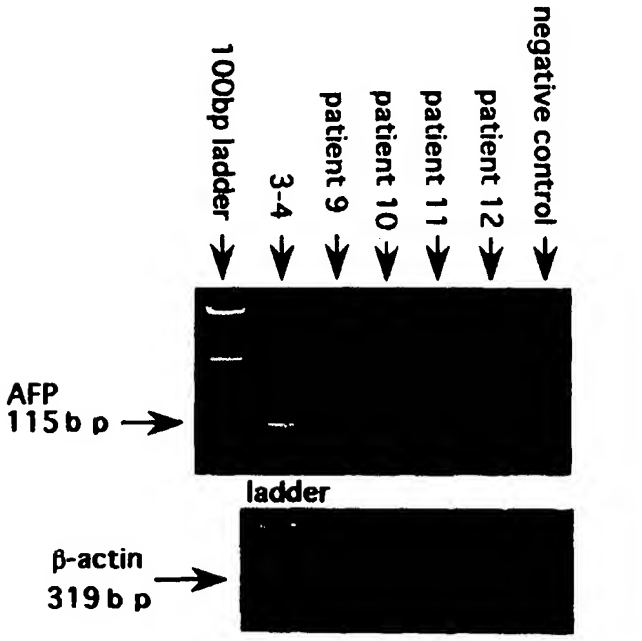


FIG. 4B

PCR amplification of virus-marker-positive patients' samples

Sequencing of the final PCR product of the positive control sample and the randomly-picked up patients' samples revealed that the amplified products were identical with the expected cDNA sequence without fail.

Discussion

In the previous decade, detecting cancer-related protein secreted into blood was the only method to know the outgrowth of primary cancer or the cancer recurrence. In recent years, trials have been reported to detect cancer cell itself in the bone marrow or in the peripheral blood via RT-PCR using specific genes. To date such studies have been conducted on melanoma cells (9), breast cancer cells (10), prostate cancer cells (11), neuroblastoma cells (12) and so on. In 1994 Matsumura et al. reported a RT-PCR system detecting AFP mRNA from HCC patients' peripheral blood after collecting nucleated cell fractions (2). According to their report, the serum AFP protein level in their patients was as high as 62738± 7031ng/ml. These HCC cells may be high-AFP-producers. And also their HCC's are exclusively large and far-advanced. For all the analysis of these advanced patients, the overall positivity within HCC patients was 52%. In order to make use of this attractive method, we hope to construct a far more sensitive system that would detect the presence of a very few number of cells and low-AFP-producing cells in the peripheral blood without consuming time for decision-making of the operative procedure and for early and direct prediction of recurrence.

First, as most HCC patients' blood is a highly infectious source of hepatitis virus, we decided to avoid the cell-fractioning procedure. In spite of having used the whole blood with the elevated RNase activity because of virus infection, we were able to obtain intact RNA enough for analysis. That is probably because of guanidine isothiocyanate-enrichment in solution D.

Second, our three-step system was able to detect the highly diluted positive control cDNA sample (Figure 2A and 2B).

Third, as we shortened each step of the PCR cycle, the blood sample obtained in the morning was processed into the final PCR product in the evening.
Using actual patients' samples, the second PCR detected AFP mRNA from 3 of a total of 7 HCC patients (43%). But the sensitivity

increased up to 71% (5 of 7) after the full three-step PCR. The serum AFP protein level of AFP mRNA-positive patients was 4325, 83, 18, 210 and 4 ng/ml, respectively. Although circulating cancer cell(s) does not necessarily adhere to and invade into patient's tissue, the presence of one patient who developed recurrence after detection of circulating AFP mRNA suggests that our system will work as a very sensitive and direct predictor of recurrence with a high probability. AFP mRNA-negative patients consisted of one preoperative patient suffering from giant HCC whose serum AFP protein level was 67213 ng/ml and one post-operative patient (serum AFP 4 ng/ml) without any recurrence even 3 months after this analysis. We also investigated patients with positive viral markers, thinking of a minor possibility of the leakage of regenerating or infected non-cancerous hepatocytes into blood (13,14). Although the number of investigated patients is four at present, neither viral-marker-positive patients nor a healthy volunteer was positive for this analysis.

When we investigated the sensitivity of our system by a mixure of HuH7 cell(s) with healthy volunteer's blood, only one cell was detected at the second PCR. The reasons for this very high sensitivity may be ① highly active AFP production by HuH7 cells (10^5pg/24hour/10^4cells even in FCS-free medium and the original patient's serum AFP level was more than 128000ng/ml)(6), ② high activity of HuH7 cells freshly detached from culture dish, ③ relatively low RNase activity of healthy volunteer's blood and ④ "career effect" of blood-derived RNA (compared to highly diluted cDNA alone). When we used hepatitis patient blood (negative for AFP mRNA by the blood alone) for dilution of HuH7 cells, one-cell-sample became faintly detectable after the second PCR and became clearly visible after the third PCR (data not shown). This may suggest that the four explanations are all tenable and that our three-step detection system is highly effective.

At present, no one can rule out the possibility of contamination of a few AFP-producing virus-infected hepatocytes or regenerating hepatocytes in the AFP mRNA-positive results, because there is no definite and widely-applicable marker to discriminate between cancer cells and non-cancerous hepatocytes. But non-cancerous hepatocytes are thought to be unable to live long without

anchoring, and the AFP mRNA positivity was zero among non-cancerous patients in the present analysis. Thus, our analysis seems to be able to tell a "high possibility" of hematogenous dissemination.

In conclusion, the three-step nested RT-PCR detection of AFP mRNA from peripheral whole blood seems to provide direct and practically useful information about hematogenous metastasis in both pre- and post-operative HCC patients.

References

1. K.OKUDA, K.KOTODA, H.OBATA, N.HAYASHI, T.HISAMITSU. Gastoro-enterology. 69 226-34 (1975)
2. M.MATSUMURA,Y.NIWA,N.KATO,Y.KOMATSU,S.SHIINA,T.KAWABE, T.KAWASE,H.TOYOSHIMA,M.IHORI,Y.SHIRATORI,M.OMATA. HEPATOLOGY 20 1418-25 (1994)
3. P.CHOMCZYNSKI, N.SACCHI.Anal.Biochem.162 156-9 (1987)
4. D.H.GILLESPIE,K.K.CUDDY,T.KOLBE,D.I.MARKS.Nature 367 390-391 (1994)
5. H.NAKABAYASHI, K.TAKETA, K.MIYANO, T.YAMANE, J.SATO. Cancer Research.45 6379-83 (1985)
6. T.MORINAGA,M.SAKAI,T.G.WEGMANN,T.TAMAOKI,Proc.Natl.Acad.Sci. USA.80 4604-8 (1983)
7. M.SAKAI,T.MORINAGA,Y.URANO. J.Biol.Chem. 260 5055-60 (1985)
8. S.A.FUQUA,N.F.FALETTE,W.L.McGUIRE,J.Natl.Cancer Inst.82 858-861 (1990)
9. B.SMITH, P.SELBY, J.SOUTHGATE, K.PITTMAN, C.BRADLEY, G. E.BLAIR. Lancet 338 1227-9 (1991)
10. H.D.DATTA, P.T.ADAMS, W.R.DROBYSKI, S.P.ETHIER, V.H.TERRY, M.S.ROTH. J.Clin.Oncol. 12 475-82 (1994)
11. D.P.Jr.WOOD, E.R.BANKS, S.HUMPHREYS, J.W.McROBERTS, V.M. RANGNEKAR.Cancer 74(9) 2533-40 (1994)
12. H.NAITO,N.KUZUMAKI,J.UCHINO,R.KOBAYASHI,T.SHIKANO,Y.ISHIKAWA Eur.J.Cancer 27(6) 762-5 (1991)
13. J.R.BLOOMER,T.A.WALDMANN,K.R.McINTIRE,G.KLATSKIN.JAMA 233 38 -41 (1975)
14. T.TAMAOKI,N.FAUSTO, In: Stein G, Stein.J (Eds.),Recombinant DNA and cell proliferation New York:Academic Press,145-168 (1984)

A4

ANNALS OF SURGERY
Vol. 229, No. 2, 255–261

Effects of Soybean Oil Emulsion and Eicosapentaenoic Acid on Stress Response and Immune Function After a Severely Stressful Operation

Katsunori Furukawa, MD, Tsuguhiko Tashiro, MD, Hideo Yamamori, MD, Kazuya Takagi, MD, Yuichi Morishima, MD, Toshiyuki Sugiura, MD, Yoshihisa Otsubo, MD, Naganori Hayashi, MD, Terumi Itabashi, MD, Wataru Sano, MD, Yasuyoshi Toyoda, MD, Hiroshi Nitta, MD, and Nobuyuki Nakajima, MD

From the First Department of Surgery, Chiba University School of Medicine, Chiba, Japan

Objective
To investigate the effects of soybean oil emulsion and oral or enteral administration of eicosapentaenoic acid (EPA) on stress response, cytokine production, protein metabolism, and immune function after surgery for esophageal cancer.

Summary Background Data
It has been reported that safflower oil, rich in n-6 polyunsaturated fatty acid (n-6 PUFA), affects the survival rate of septic animals and decreases the immune function. It has also been reported that the administration of fish oil, in contrast, reduces these stress responses and stress-induced immunosuppression. In humans, the effects of soybean oil emulsion and the administration of EPA on stress response and immune function after surgery have not been established.

Methods
Patients who underwent esophagectomy with thoracotomy were divided into three groups. Seven patients were fed by total parenteral nutrition (TPN) with soybean oil emulsion, which accounted for 20% of total calories. Seven patients were given oral or enteral administration of 1.8 g/day EPA, in addition to TPN with soybean oil emulsion. Nine patients served as the control group; these patients received fat-free TPN. Serum interleukin-6 (IL-6), C-reactive protein, concanavalin A (con A)- or phytohemagglutinin (PHA)-stimulated lymphocyte proliferation, natural killer cell activity, and stress hormones were measured.

Results
The postoperative level of serum IL-6 was significantly higher in the group receiving soybean oil emulsion than in the fat-free group. Oral or enteral supplementation of EPA with soybean oil emulsion significantly reduced the level of serum IL-6 compared with the patients receiving soybean oil emulsion. Con A- or PHA-stimulated lymphocyte proliferation decreased significantly on postoperative day 7 in all groups of patients. The supplementation of EPA with soybean oil emulsion significantly improved the lymphocyte proliferation and natural killer cell activity on postoperative day 21 compared with the group receiving soybean oil emulsion.

Conclusions
Soybean oil emulsion amplifies, and the supplementation of EPA reduces, the stress response and stress-induced immunosuppression.

Linoleic acid, one of the n-6 polyunsaturated fatty acids (PUFA), is the precursor of arachidonic acid, which in turn gives rise to the dienoic prostaglandins and leukotrienes.[1–6] It has been shown that levels of prostaglandin (PG) E_2, derived from arachidonic acid, increase in a stressed state and suppress immune function. Therefore, n-6 PUFA may adversely affect inflammatory and immunologic responses in critically ill patients. Animal experiments have indicated that safflower oil, rich in n-6 PUFA, enhances the stress response and stress-induced immunosuppression.[7–12] The fat emulsion currently used in clinical practice is limited to

Correspondence: Katsunori Furukawa, MD, First Department of Surgery, Chiba University School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260, Japan.
Accepted for publication July 24, 1998.

Table 1. FATTY ACID COMPOSITION OF SOYBEAN OIL EMULSION

Myristic acid	(C14:0)	NR
Palmitic acid	(C16:0)	12.4%
Palmitoleic acid	(C16:1)	NR
Stearic acid	(C18:0)	4.4%
Oleic acid	(C18:1)	23.9%
Linoleic acid	(C18:2)	51.6%
Linolenic acid	(C18:3)	7.7%
Arachidonic acid	(C20:4)	1.6%
EPA	(C20:5)	<0.1%

NR, not reported; EPA, eicosapentaenoic acid

soybean oil emulsion containing >50% of linoleic acid (Table 1). The perioperative use of soybean oil emulsion may amplify the stress response and stress-induced immunosuppression in critically ill patients. However, n-3 PUFA, the other type of essential fatty acid, decreases the synthesis of PGE_2 and generates the biologically less active metabolite PGE_3 from eicosapentaenoic acid (EPA).[1,4–6] Administration of n-3 PUFA may reduce the adverse effects of n-6 PUFA.

In this study, we investigated the effects of soybean oil emulsion and EPA on stress response and immune function after surgery for esophageal cancer.

PATIENTS AND METHODS

Patients and Clinical Protocol

This prospective, randomized, double-blind protocol was approved by the Ethics Committee of Chiba University School of Medicine. Twenty-three patients were enrolled into the study. Informed written consent was obtained from all patients. They underwent esophagectomy with thoracotomy and three-field lymph node dissection. This was followed by reconstructive surgery using a gastric tube or colon replacement by the retrosternal route. According to our previous study,[13] they were fed exclusively by total parenteral nutrition (TPN), which provided 1.5 g of protein/kg/day and 40 kcal/kg/day from the seventh day before surgery to postoperative day (POD) 14. The rate of parenteral nutrition started at 20 kcal/kg/day and was gradually increased according to patient tolerance to a target rate that would deliver 40 kcal/kg/day.

The patients were divided into three groups. Nine control patients received fat-free TPN (group I). Seven patients received TPN with a soybean oil emulsion (Intralipid), which accounted for 20% of the total calories (group II). Seven patients were given orally or enterally 1.8 g/day of EPA in addition to TPN with soybean oil emulsion (group III). After POD 14, TPN was gradually switched to enteral feeding. This consisted of 17.7% medium-chain triglyceride and 4% linoleic acid. Soybean oil emulsion continued to be administered to groups II and III and EPA to group III until POD 21. The soybean oil emulsion contained 51.6% linoleic acid and 7.7% linolenic acid (see Table 1).

All patients had normal hepatic and renal function and were not diabetic. They did not receive preoperative chemoradiation therapy.

Blood and Urine Sampling

Interleukin-6 (IL-6), C-reactive protein (CRP), and glucagon can be used to gauge the intensity of the injury stress response. Concanavalin A (con A)- and phytohemagglutinin (PHA)-stimulated lymphocyte proliferation and natural killer (NK) cell activity are indices of cell-mediated immunity, and the rapid turnover proteins and nitrogen studies give some index of the amount of acute protein breakdown that was occurring.

The serum concentration of IL-6 was determined before surgery; 1, 2, and 12 hours after surgery; and 3 and 10 days after surgery. Glucagon and CRP were determined before surgery and on POD 1, 3, and 10. Con A- or PHA-stimulated lymphocyte proliferation and NK cell activity were determined before surgery and on POD 7 and 21. Rapid turnover proteins were measured before surgery and on POD 1, 3, 10, and 21. Urine was collected daily, and the daily nitrogen losses were calculated by multiplying the nitrogen concentration measured in urine by the total amount of ultradiafiltrate produced over a 24-hour period and by adding to this the standard estimate for insensible nitrogen losses (15 mg/kg/day). Daily nitrogen balance was estimated as the difference between intake and losses. The daily nitrogen balance was assessed through POD 7.

Laboratory Analyses

The concentration of serum IL-6 was assayed using a commercial human cytokine enzyme-linked immunosorbent assay kit (Amersham, UK). The cytokine assay was standardized by inclusion of a titration of the appropriate purified recombinant cytokine of known concentration. The absorbance of the sample was determined with 450 nm as the primary wave length.

Fasting glucagon was measured in plasma from venous blood that was mixed with Trasyrol immediately after collection in EDTA tubes and centrifuged at 4°C. Glucagon concentrations were assayed using a double-antibody ^{125}I radioimmunoassay kit (Daiich RI, Tokyo, Japan).

Mononuclear cells were separated from venous blood by density gradient centrifugation on Conray/Ficoll. Mononuclear cells removed from the interface were washed two times in phosphate-buffered saline. 1×10^5 cells in a final volume of 200 μl of RPMI 1640 with 10% fetal calf serum were plated in microtiter wells. The cells were incubated with the T-cell mitogens, Con A and PHA, in final concentrations of 10 μg/ml. Assays were performed in duplicate, and unstimulated background control cultures were incubated with every assay. Cells were incubated at 37°C for 64

Table 2. DEMOGRAPHIC, PREOPERATIVE, AND SURGICAL DATA

	Group I	Group II	Group III
Number of patients	9	7	7
Age (years)	64 ± 5	58 ± 4	61 ± 3
Sex (men/women)	8/1	6/1	6/1
Albumin (g/dl)	3.8 ± 0.1	4.0 ± 0.1	3.9 ± 0.2
Prealbumin (mg/dl)	29 ± 1	26 ± 1	29 ± 2
Transferrin (mg/dl)	224 ± 12	260 ± 8	245 ± 23
Con A (SI)	142 ± 24	130 ± 43	182 ± 44
PHA (SI)	175 ± 37	149 ± 44	214 ± 45
NK (%)	29 ± 5	34 ± 7	39 ± 9
Operation time (minutes)	592 ± 42	595 ± 45	642 ± 48
Blood loss (g)	979 ± 213	961 ± 444	810 ± 336

Data are expressed as means ± SEM.
p = not significant among all categories
Con A = con A-stimulated lymphocyte proliferation; PHA = PHA-stimulated lymphocyte proliferation; NK = natural killer cell activity; SI = stimulation index.

hours with an 8-hour pulse labeling with ^{3}H-thymidine, 0.25 μCi/well. Cells were harvested onto glass-fiber filters, and ^{3}H-thymidine content and hence proliferation were determined by liquid scintillation counting. Stimulation indices were calculated by dividing the counts per minute (cpm) of ^{3}H-thymidine in mitogen-stimulated cells by the cpm in cells cultured without mitogens.

Natural killer cell activity was measured by a standard 4-hour ^{51}Cr-release assay, using K 562 as target cells at effector/target cell (E/T) ratios of 20:1. NK cell activity, expressed as percentage cytotoxicity, was calculated by the following formula: % cytotoxicity = ([experimental release − spontaneous release]/[total release − spontaneous release]) × 100.

C-reactive protein and rapid turnover proteins such as transferrin, retinol binding protein, and prealbumin were measured by radial immunodiffusion assay. This technique allows quantitative determination of human plasma proteins after 24 hours of diffusion. Serum containing the protein to be tested is placed in a well on the test plate. The area of the resulting antibody–antigen precipitin zone is directly related to the concentration of the substance placed in the plate well.[14]

Urinary concentrations of nitrogen were measured using a chemiluminescence method. Briefly, nitrogen in biologic samples is oxidized at 1100°C, yielding nitric oxide. On contact with ozone, a metastable nitrogen dioxide is generated that emits photons on decay. The intensity of the emitted light is proportional to the nitrogen content of the sample.[15]

Statistical Analyses

All values are expressed as mean ± standard error of the mean. Statistical analyses were performed using Fisher's protected least significant difference when the overall analysis of variance was significant. $P < 0.05$ was considered significant.

RESULTS

The clinical details of all patients studied are shown in Table 2. There were no statistical differences among these three groups in age, sex ratio, baseline nutritional date, preoperative cell-mediated immunity, blood loss, and operating time.

The profiles of IL-6 production are illustrated in Figure 1. In group II, serum IL-6 levels peaked 2 hours after surgery (569 ± 111 pg/ml). Serum IL-6 levels in group II were statistically higher than in group I (321 ± 21 pg/ml) at 2 hours after surgery ($p < 0.05$). Serum IL-6 levels were significantly lower in group 3 (195 ± 54 pg/ml) than in group II at 2 hours after surgery ($p < 0.01$). A statistically significant difference between the two groups was maintained for the duration of the study ($p < 0.01$ at 10 days after surgery, $p < 0.05$ at 1 and 12 hours and 3 days after surgery). Serum IL-6 levels in group III returned to preoperative levels on POD 10.

The profiles of CRP are illustrated in Figure 2. CRP levels peaked on POD 3 in all groups. On POD 3, the CRP levels in group III (13 ± 2.1 mg/dl) were significantly lower ($p < 0.05$) than that in group II (22.3 ± 2.1 mg/dl).

The profiles of glucagon are illustrated in Figure 3. In all groups, glucagon peaked on POD 1 and declined in the subsequent days. On POD 1, the glucagon levels in group III (188 ± 16 pg/ml) were significantly lower ($p < 0.05$) than those in group II (298 ± 52 pg/ml). A statistically significant difference between the two groups was maintained throughout the study ($p < 0.05$).

Con A-stimulated lymphocyte proliferation (stimulation index) decreased significantly on POD 7 compared with the preoperative value in all groups ($p < 0.05$) (Fig. 4). On POD 21, Con A-stimulated lymphocyte proliferation in

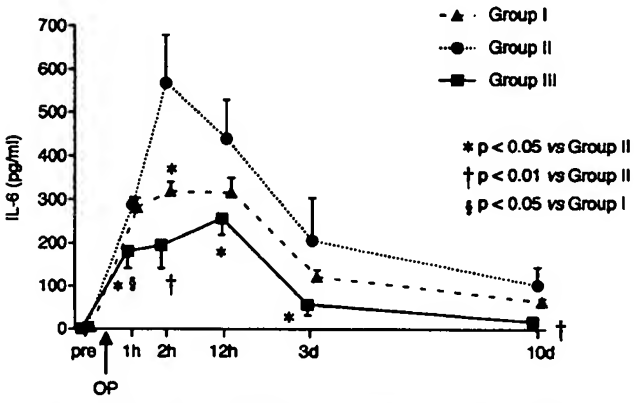


Figure 1. Serum concentration of interleukin-6 (IL-6). Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group 2; † $p < 0.01$ *vs.* group 2; § $p < 0.05$ *vs.* group 1. pre, before surgery; OP, operation; h, postoperative hours; d, postoperative days.

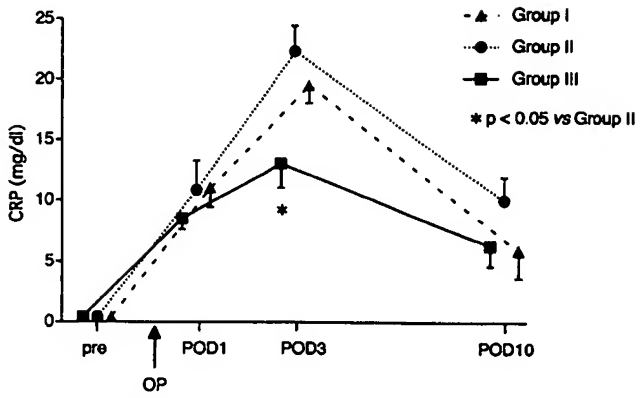


Figure 2. Serum concentration of C-reactive protein. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group II. pre, before surgery; OP, operation; POD, postoperative days.

group III (200 ± 33) was statistically higher ($p < 0.05$) than on POD 7 (65 ± 26), and returned to preoperative levels (182 ± 44). It was also significantly higher than in the other groups ($p < 0.05$).

The stimulation index of PHA-stimulated lymphocyte proliferation also decreased significantly on POD 7 compared with the preoperative value in groups II and group III ($p < 0.05$). On POD 21, PHA-stimulated lymphocyte proliferation in group III (242 ± 36) was statistically higher ($p < 0.05$) than on POD 7 (81 ± 40), and returned to preoperative levels (214 ± 45). It was also statistically significantly higher than that in group I ($p < 0.05$).

On POD 21, NK cell activity in group III (49 ± 8%) was significantly higher ($p < 0.05$) than in group II (28 ± 7%) (Fig. 5).

Transferrin, retinol binding protein, and prealbumin levels decreased significantly after surgery, but there were no significant differences among the three groups (Fig. 6). Although a gradual reduction in cumulative nitrogen balance was observed after surgery, there were no statistically significant differences among the three groups (Fig. 7).

DISCUSSION

It has been generally accepted that supplementation of adequate amounts of lipid is useful in critically ill patients. Lipids are inexpensive, provide essential fatty acids, and reduce the glucose load. Critically ill patients have been shown to metabolize lipid normally or even at an accelerated rate.[16]

Commercially available fat emulsion is limited to soybean oil emulsion, which contains >50% linoleic acid. Overactivation of the arachidonic acid pathway through the provision of excessive amounts of the precursor linoleic acid by soybean oil emulsion has been suggested to have deleterious effects in critically ill patients.

We have investigated the effect of intravenous n-6 fat emulsion using safflower oil on nitrogen retention, protein kinetics, cytokine production, and cell-mediated immune function in burned rats. We concluded that the administration of n-6 PUFA-enriched fat emulsion increased levels of serum cytokines such as tumor necrosis factor-alpha and IL-6, IL-8, and IL-10 in burned rats. We also demonstrated that nitrogen retention was affected and delayed-type hypersensitivity was suppressed by the administration of safflower oil emulsion in burned rats.[7–10]

Mochizuki et al[11] have reported adverse effects when safflower oil emulsion accounted for approximately 30% to 50% of nonprotein calories. These effects negatively impacted muscle mass, nitrogen balance, and serum protein in burned guinea pigs. Alexander et al[12] have found that enteral administration of safflower oil increased the release of PGE_2 from splenic macrophage and decreased delayed-type hypersensitivity in burned guinea pigs.

According to our clinical experiment, soybean oil emulsion increased the level of serum IL-6. Nitrogen balance tended to be aggravated when compared with the patients given soybean oil emulsion. Cell-mediated immune function was suppressed after surgery for esophageal surgery, which is one of the most severe surgical procedures. The administration of soybean oil emulsion did not result in significant differences in cell-mediated immune function and NK cell activity when compared with the group given fat-free TPN.

The other type of essential fatty acid, n-3 PUFA, has been reported to modulate inflammatory and immune responses in animals and humans. We performed an animal experiment to investigate the effect of fish oil emulsion on protein metabolism, inflammatory response, cytokine production, and immune function. We observed that the administration of fish oil emulsion decreased the serum levels of IL-6, IL-8, IL-10, and tumor necrosis factor-alpha when compared with the burned rats fed safflower oil emulsion. Protein metabolism was also improved by the administration of fish oil emulsion.[7–10]

Alexander et al[12] have reported that the administration of fish oil significantly improved weight loss, promoted skeletal muscle mass, decreased energy expenditure, and in-

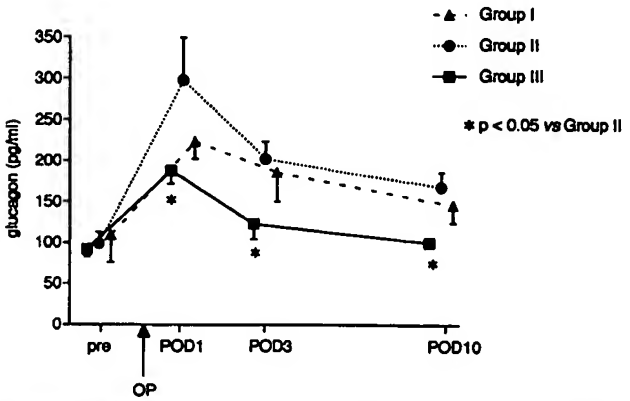


Figure 3. Serum concentration of glucagon. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group II. pre, before surgery; OP, operation; POD, postoperative days.

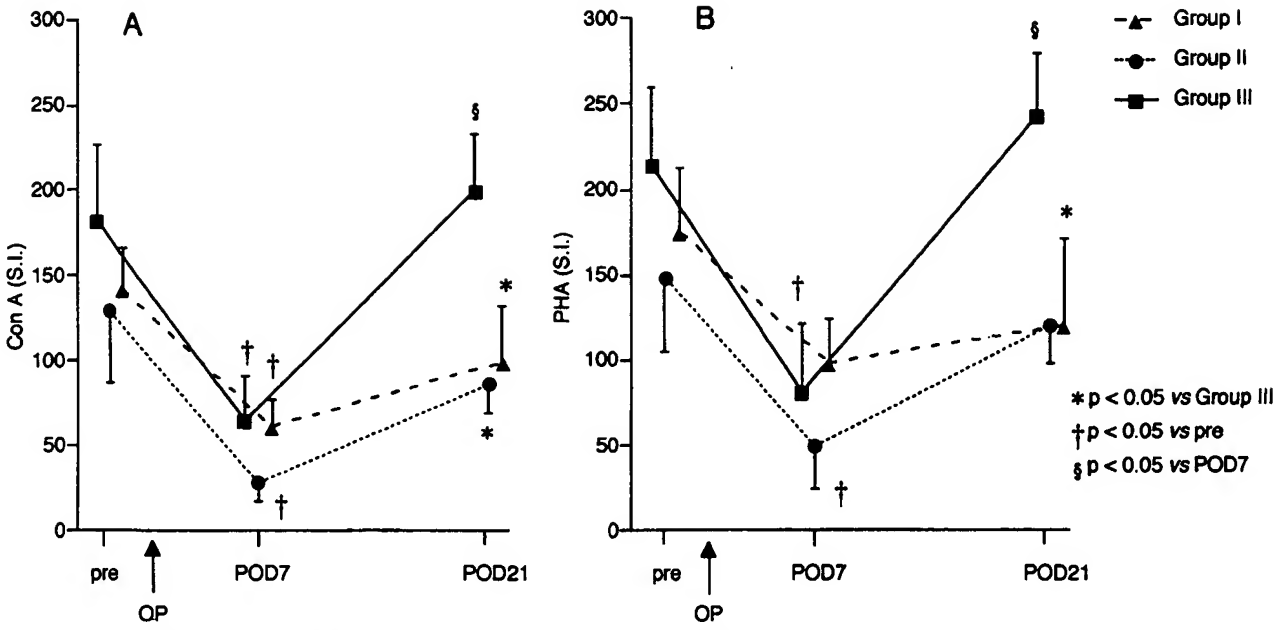


Figure 4. (A) Concanavalin A-stimulated lymphocyte proliferation. (B) Phytohemagglutinin-stimulated lymphocyte proliferation. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group III; † $p < 0.05$ *vs.* pre; § $p < 0.05$ *vs.* POD 7. pre, before surgery; OP, operation; POD, postoperative days; S.I., stimulation index.

creased delayed-type hypersensitivity. Saito et al[17] have reported that resting metabolic expenditures and immunologic responses were improved when fish oil was substituted for linoleic acid or when indomethacin was given in conjunction with a safflower oil-based lipid diet.

In humans, the effects of n-3 PUFA have been investigated in patients with chronic inflammatory disease, such as rheumatoid arthritis, psoriasis, multiple sclerosis, or ulcerative colitis.[18–22] Meydani et al[23] found that n-3 fatty acid supplementation in both young and older women suppressed cytokine production. Endres et al[24] found that n-3 fatty acid supplementation reduced the synthesis of IL-1 and tumor necrosis factor by mononuclear cells. However, the effects of n-3 PUFA have not been clarified in critically ill patients with multiple trauma, extensive burn injuries, or sepsis.

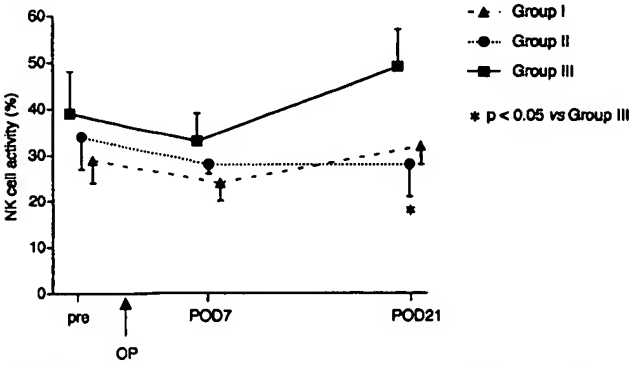


Figure 5. Natural killer cell activity. Data are expressed as means ± SEM. * $p < 0.05$ *vs.* group III. pre, before surgery; OP, operation; POD, postoperative days.

In this study, oral or enteral administration of EPA significantly reduced the postoperative serum IL-6 level compared with the group given fat-free TPN with soybean oil emulsion. Postoperative stress-induced immunosuppression was significantly reduced by the addition of EPA, which accounted for only 1% of total caloric intake, even though the same amount of soybean oil emulsion was infused in this study group.

The immunologic effects of n-3 PUFA are not uniform. Yoshino and Ellis[25] have found that fish oil-derived fatty acids modulated chronic inflammation and cell-mediated immunologic reactions by reducing the synthesis of arachidonic acid metabolites in rats. Calder[26] found that supplementation of the diet of healthy humans with fish oil capsules suppressed lymphocyte proliferation and IL-2 production. Endres et al[27] found that dietary n-3 fatty acids decreased the production of IL-2 and reduced mononuclear cell proliferation in healthy humans. All of these studies were performed in nonstressed patients. The improvements in immune function in a stressed patients obtained from both our experimental and clinical studies demonstrate the possibility that the effect of n-3 PUFA on immune function in a stressed state may be different from that in a nonstressed state, as reported before.[7–10] It is widely known that cell-mediated immune functions are affected by severe stress. The data from our studies indicate that n-3 PUFA reduced rather than increased stress-induced immunosuppression.

Several reports exist regarding the mechanism underlying the effects of n-3 PUFA. Alterations in the type of arachidonic acid metabolites produced during stimulation of the

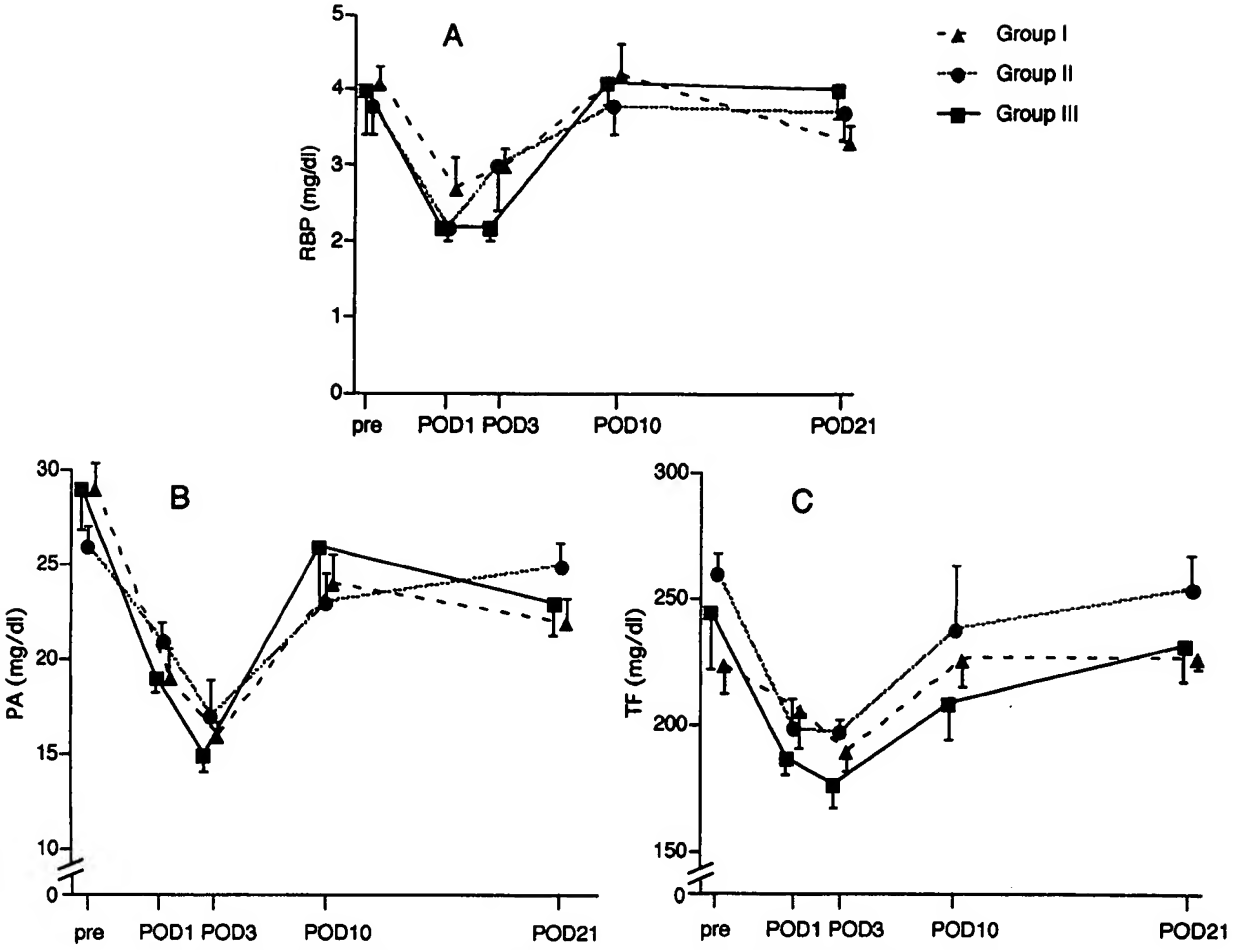


Figure 6. (A) Serum concentration of retinol binding protein. (B) Serum concentration of prealbumin. (C) Serum concentration of transferrin. Data are expressed as means ± SEM. pre, before surgery; POD, postoperative days.

mononuclear cells may explain, in part, the changed production of IL-6 in the cytokine network. A possible mechanism for the changed IL-6 production due to n-6 and n-3 PUFA is altered synthesis of leukotriene B4 and generation of the biologically less active metabolite leukotriene B5 from EPA.[1-6,24] It is well known that levels of PGE_2, derived from arachidonic acid, increase in a stressed state and suppress immune function. Thus, a possible mechanism for reduced immunosuppression by the administration of n-3 PUFA in a stressed state is the decreased synthesis of PGE_2 and the generation of the biologically less active metabolite PGE_3 from EPA.[1,4-6] Another possible mechanism for the effects of n-6 and n-3 PUFA is the result of changes in membrane fluidity,[28] as well as the changes in the release of membrane-associated intracellular messengers, such as phosphate inositol and diacylglycerol.[29,30] Chang et al[31] have suggested that fish oil enhances tumor necrosis factor-alpha mRNA expression of macrophage at the transcriptional level. Details of the exact mechanism remain to be worked out, however.

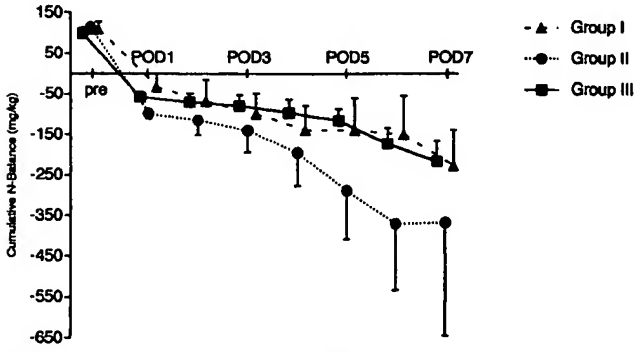


Figure 7. Cumulative nitrogen balance. Data are expressed as means ± SEM. pre, before surgery; POD, postoperative days.

Recent studies, as well as the experimental data from our laboratory and from others, suggest that n-3 PUFA has beneficial effects in critically ill patients in both stress response and immune function. However, n-6 PUFA is still an essential component of cell membrane phospholipids and is critical to the maintenance of cellular functions, particularly in critically ill patients. Excessive levels of n-6 PUFA

without n-3 PUFA have been shown to increase the production of cytokines and promote immunosuppression. It was also found that the addition of EPA to soybean oil emulsion reduced the stress response, improved protein metabolism, and prevented stress-induced immunosuppression.

References

1. Granstrom E. The arachidonic acid cascade: the prostaglandins, thromboxanes and leukotriene. Inflammation 1984; 8:515–524.
2. Lee TH, Hoover RL, Williams JD, et al. Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. N Engl J 1985; 312:1217–1224.
3. Lee TH, Sethi T, Crea AEG, et al. Characterization of leukotriene B3: comparison of its biological activities with leukotriene B4 and leukotriene B5 in complement receptor enhancement, lysozyme release and chemotaxis of human neutrophils. Clin Sci 1988; 74:467–475.
4. Kinsella JE, Lokesh B, Broughton S, Whelan J. Dietary polyunsaturated fatty acids and eicosanoids: potential effects on the modulation of inflammatory and immune cells: an overview. Nutrition 1990; 6:24–44.
5. Needleman P, Raz A, Minkes MS, et al. Triene prostaglandins: prostacyclin and thromboxane biosynthesis and unique biological properties. Proc Natl Acad Sci USA 1979; 76:944–948.
6. Surette ME, Whelan J, Lu G, et al. Dietary n-3 polyunsaturated fatty acids modify Syrian hamster platelet and macrophage phospholipid fatty acyl composition and eicosanoid synthesis: controlled study. Biochim Biophys Acta 1995; 1255:185–191.
7. Tashiro T, Hayashi N, Yamamori H, et al. N-6 fat emulsion aggravates and n-3 fat emulsion improves protein metabolism in burned rats. JPEN 1996; 20(Suppl):377.
8. Hayashi N, Tashiro T, Yamamori H, et al. N-6 fat emulsion increases and n-3 fat emulsion reduces cytokine production in burned rats. JPEN 1996; 20(Suppl):382.
9. Hayashi N, Tashiro T, Yamamori H, et al. Effect of n-3 fat emulsion on immune response and relation to granulocyte-macrophage colony-stimulating factor (GMCSF) in burned rats receiving total parenteral nutrition (TPN). Jpn J Surg Metab Mutr 1997; 31:225–232.
10. Hayashi N, Tashiro T, Yamamori H, et al. The effect of n-3 fat emulsion on fatty acid metabolism and cytokine release in burned rats receiving TPN. Jpn J Surg Metab Mutr 1996; 30:283–290.
11. Mochizuki H, Trocki O, Dominioni L, et al. Optimal lipid content for enteral diets following thermal injury. JPEN 1984; 8:638–646.
12. Alexander JW, Saito H, Ogle CK, Trocki O. The importance of lipid type in the diet after burn injury. Ann Surg 1986; 204:1–8.
13. Tashiro T, Yamamori H, Takagi K, et al. Effect of protein dose on nitrogen retention in postoperative patients receiving total parenteral nutrition. Surg Res Comm 1996; 18:231–238.
14. Mancini G, Carbonera AO, Heremans JF. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry 1965; 2:235–254.
15. Ward MWN, Owens CWI, Rennie MJ. Nitrogen estimation in biological samples by use of chemiluminescence. Clin Chem 1980; 26:1336–1339.
16. Carpenter YA, Askanazi J, Elwyn DH, et al. Effects of hypercaloric glucose infusion on lipid metabolism in injury and sepsis. J Trauma 1979; 19:649–654.
17. Saito H, Trocki O, Heyd T, Alexander JW. Effect of dietary unsaturated fatty acids and indomethacin on metabolism and survival after burn. Abstracts of the American Burn Association 1985; 17:27.
18. Chandrasekar B, Fernandes G. Decreased pro-inflammatory cytokines and increased antioxidant enzyme gene expression by w-3 lipid in murine lupus nephritis. Biochem Biophys Res Commun 1994; 200: 893–898.
19. Soyland E, Lea T, Sandstad B, et al. Dietary supplementation with very-long-chain n-3 fatty acids in man decreases expression of the interleukin-2 receptor (CD25) on mitogen-stimulated lymphocytes from patients with inflammatory skin disease. Eur J Clin Inves 1994; 24:236–242.
20. Kremer JM, Lawrence DA, Petrillo GF, et al. Effects of high-dose fish oil on rheumatoid arthritis after stopping nonsteroidal antiinflammatory drugs. Clinical and immune correlates. Arthritis Rheumatism 1995; 38:1107–1114.
21. Esperson GT, Grunnet N, Lervang HH, et al. Decreased interleukin-1 beta levels in plasma from rheumatoid arthritis patients after dietary supplementation with n-3 polyunsaturated fatty acids. Clin Rheumatol 1992; 11:393–395.
22. Gallai V, Sarchielli P, Trequattrini A, et al. Cytokine secretion and eicosanoid production in peripheral blood mononuclear cells of MS patients undergoing dietary supplementation with n-3 polyunsaturated fatty acids. J Neuroimmunol 1995; 56:143–153.
23. Meydani SN, Endres S, Wood MM, et al. Oral (n-3) fatty acid supplementation suppresses cytokine production and lymphocyte proliferation: comparison between young and older women. J Nutr 1991; 121:547–555.
24. Endres S, Ghorbani R, Kelley VE, et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. N Engl J Med 1989; 320:265–271.
25. Yoshino S, Ellis EF. Effect of a fish-oil-supplemented diet on inflammation and immunological process in rats. Int Arch Allergy Appl Immunol 1987; 84:233–240.
26. Calder PC. The effects of fatty acids on lymphocyte function. Braz J Med Res 1993; 26:901–917.
27. Endres S, Meydani SN, Ghorbani R, et al. Dietary supplementation with n-3 fatty acids suppresses interleukin-2 production and mononuclear cell proliferation. J Leukoc Biol 1993; 54:599–603.
28. Stubbs CD, Smith AD. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984; 779:89–137.
29. Billar TR, Bankey PE, Svingen BA, et al. Fatty acid intake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. Surgery 1988; 104:343–349.
30. Fowler KH, McMurray DN, Fan YY, et al. Purified dietary n-3 polyunsaturated fatty acids alter diacylglycerol mass and molecular species composition in concanavalin A-stimulated murine splenocytes. Biochim Biophys Acta 1993; 1210:89–96.
31. Chang HR, Arsenijevic D, Vladoianu IR, et al. Fish oil enhances macrophage tumor necrosis factor-alpha mRNA expression at the transcriptional level. Metabolism 1995; 44:800–805.

A5

Bone Marrow Transplantation (2001) 27, 1095–1100

www.nature.com/bmt

Immunobiology

Oligoclonal expansion of CD4 CD28 T lymphocytes in recipients of allogeneic hematopoietic cell grafts and identification of the same T cell clones within both CD4 CD28 and CD4 CD28 T cell subsets

M Hirokawa[1], T Horiuchi[1], Y Kawabata[1], A Kitabayashi[1], H Saitoh[1], Y Ichikawa[1], T Matsutani[2], T Yoshioka[2], Y Tsuruta[2], R Suzuki[2] and AB Miura[1]

[1]*Department of Internal Medicine III, Akita University School of Medicine, Akita; and* [2]*Department of Immunology, Shionogi Institute for Medical Science, Osaka, Japan*

Summary:

Recipients of allogeneic bone marrow grafts have clonally expanded CD8 CD28 T lymphocytes during the early period after transplantation, which leads to skewing of T cell receptor (TCR) repertoires. Here, we have addressed the question of whether clonal expansion of CD28 T cells is also observed in CD4 T lymphocytes after human allogeneic hematopoietic cell transplantation. We found that the fraction of T cells lacking CD28 expression in the CD4 subset was increased after transplantation, and expanded CD4 CD28 T lymphocytes carrying certain TCRBV subfamilies showed limited TCR diversity. In order to further study the ontogeny of CD4 CD28 T cells, we analyzed the complementarity-determining region 3 (CDR3) of the TCR-β chain of CD4 CD28 and CD4 CD28 cells. We identified the same T cell clones within both CD4 CD28 and CD4 CD28 T cell subsets. These results suggest that both subsets are phenotypic variants of the same T cell lineage. *Bone Marrow Transplantation* (2001) **27**, 1095–1100.

Keywords: TCR-β; CDR3; clone; CD4; CD28

The CD28 molecule is a disulfide-linked homodimer expressed on the surface of T cells and it binds to the natural ligand B7 family members, CD80/CD86, expressed on antigen-presenting cells, resulting in costimulation of T cell activation.[1] In humans, all thymocytes and the vast majority of peripheral blood T cells express CD28 at birth. An increase of the proportion of CD8 T cells lacking CD28 expression has been demonstrated in the aged population,[2] HIV-infected individuals[3,4] and bone marrow transplant recipients.[5,6] In contrast, in healthy individuals, only a few percent of CD4 T cells lack CD28,[3] and expansion of CD4 CD28 T cells is scarcely reported in rheumatoid arthritis patients.[7,8]

Correspondence: Dr M Hirokawa, Akita University School of Medicine, Department of Internal Medicine III, 1-1-1 Hondo, Akita 010-8543, Japan
Received 19 January 2001; accepted 13 March 2001

Loss of CD28 expression has recently been implicated in lymphocyte senescence.[9,10] We have previously reported the oligoclonal expansions of CD8 CD28 T lymphocytes in recipients of allogeneic bone marrow grafts and the demonstration of identical T cell clones within both CD8 CD28 and CD8 CD28 T cell subsets.[11] These results suggest that clonally expanded CD8 CD28 T cells after allogeneic hematopoietic cell transplantation (HCT) are derived from the CD8 CD28 T cell subset, presumably by an antigen-driven mechanism. In the present study, we have addressed the question of whether clonal expansion of CD4 CD28 T cells is also observed in recipients of allogeneic hematopoietic cell grafts and whether these cells are the descendents of CD4 T cells expressing CD28.

Materials and methods

Patients

Informed consent was obtained from the patients before blood samples were collected. All the patients were conditioned with myeloablative chemoradiotherapy, mostly consisting of fractionated total body irradiation (12 Gy in six fractions) and cyclophosphamide (60 mg/kg/day for 2 days), followed by infusion of allogeneic marrow or blood stem cell grafts from HLA-matched donors. All the patients received cyclosporin A and short-term methotrexate for prophylaxis of acute graft-versus-host disease (GVHD).[12] Engraftment was achieved in all patients, and confirmed by recovery of hematopoiesis and the presence of donor-derived sex chromosomes or mismatched antigens on red cells. All patients were seropositive for cytomegalovirus (CMV). Patients were monitored for CMV infection by weekly CMV antigenemia assays from when the granulocyte count reached 500/μl until day 100 after transplantation. Patients who were positive for CMV antigenemia assay received prophylactic ganciclovir (5 mg/kg/day, 3 days a week) from the time the granulocyte count became greater than 1000/μl.

Flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by the Ficoll/Conray gradient centrifugation method. PBMCs were stained with FITC- or PE-conjugated monoclonal antibodies and analyzed using a flow cytometer (Cytron Absolute, Ortho Diagnostics, Tokyo, Japan). Monoclonal antibodies used in this study were as follows: anti-CD3 (SK7, IgG1; Becton Dickinson, San Jose, CA, USA); anti-CD4 (MT310, IgG1; DAKO, Glostrup, Denmark); anti-CD8 (DK25, IgG1; DAKO); anti-CD28 (CD28.2, IgG1; Beckman Coulter, Fullerton, CA, USA); and control mouse IgG (X40, IgG1; DAKO). In some experiments, CD4 CD28 , CD4 CD28 , CD8 CD28 and CD8 CD28 T cell subsets were collected by FACS sorting. Each population was >98% pure.

TCRBV repertoire analysis

Analysis of the T cell receptor β-chain variable region (TCRBV) repertoire was performed by an adapter ligation PCR-based microplate hybridization assay, as reported previously.[13,14] Briefly, total RNA was extracted from PBMCs and converted to double-strand cDNA using a SuperScript cDNA synthesis kit (BRL, Bethesda, MD, USA). The P10EA/P20EA adapters were ligated to the 5′ end of cDNA prepared from PBMCs, and PCR was performed using a biotinylated TCRCB-specific primer and a P20EA primer. Biotinylated PCR products were hybridized with immobilized TCRBV-specific primers in 96-well microtiter plates. Subsequently, alkaline phosphatase-conjugated streptavidin was added to each well and a colorimetric assay was performed. Skewing of the TCR repertoire was defined as a significant increase of TCRBV subfamilies to a greater percentage than the mean plus three standard deviations (s.d.) of 20 healthy individuals, and exceeded 5% of total circulating blood T cells.[14]

PCR amplification and CDR3 size distribution analysis of the TCR-β chain

The procedure for CDR3 size analysis (spectratyping) for the TCR-β chain has been described elsewhere.[15,16] Total RNA was extracted from PBMCs using an RNeasy Total RNA Kit (Qiagen; Hilden, Germany) and was used for first-strand cDNA synthesis with an oligo-dT primer (First-Strand cDNA Synthesis Kit; Amersham Pharmacia Biotech, Uppsala, Sweden). Aliquots of the cDNA were amplified with a Vβ-specific primer and a Cβ-specific primer. Primer sequences were described previously.[16,17] PCR amplification was performed for 40 cycles in a 20 μl reaction mixture containing 0.2 μM of each primer and 0.5 U of Taq polymerase (TaKaRa, Osaka, Japan). Conditions for the PCR were as follows: denaturation at 94 for 1 min, annealing at 55 C for 1 min, and extension at 72 for 1.5 min. Following the 40 cycles of PCR, an additional extension at 72 C for 15 min was performed. The PCR buffer was 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 0.2 mM of each dNTP.

Aliquots (4 μl) of the unlabeled Vβ–Cβ PCR products were subjected to one cycle of elongation (runoff reaction) with a FAM-labeled nested Cβ primer (FAM-CB3) under the following conditions: denaturation at 94 for 2 min, annealing at 55 for 1 min, and extension at 72 for 15 min. The reaction buffer was the same as that described above. The labeled PCR products were mixed with the size marker (GeneScan-500 TAMRA; Applied Biosystems, Warrington, UK), and loaded on to a 5% polyacrylamide sequencing gel for determination of size and fluorescence intensity using an automated DNA sequencer (ABI 377; Perkin-Elmer Applied Biosystems, Foster, CA, USA). Data were analyzed using GeneScan software (Perkin-Elmer Applied Biosystems).

Sequencing of CDR3 region in the TCR-β chain

PCR products of the TCR-β chain were cloned into the PCR2.1 TA cloning vector (Invitrogen, Carlsbad, CA, USA) and sequenced using a Big-Dye Terminator Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems).[18] Sequence analysis was performed using an Applied Biosystems 377A automatic DNA sequencer.

Results

Oligoclonal expansion of CD4 CD28⁻ T cells after allogeneic hematopoietic cell transplantation

We have previously reported the oligoclonal expansion of CD8 T cells lacking CD28 expression during an early period after allogeneic bone marrow transplantation, which imposes skewing upon the post-transplant TCR repertoire of circulating blood T cells.[11] Moreover, we have demonstrated the presence of identical clones within both CD8 CD28 and CD8 CD28 T cell subsets.[11] These results suggest that CD8 CD28 and CD8 CD28 T cells are phenotypic variants of the same lineage. If chronic antigenic stimulation causes oligoclonal expansion of CD8 CD28 T cells after transplantation, it is likely that clonal expansion of CD4 CD28 T lymphocytes as well as CD8 CD28 T cells occurs.

We first examined the CD28 expression of CD4 T lymphocytes in 23 patients who received allogeneic bone marrow grafts. There was a marked increase of the CD28 T cell fraction in CD4 T cells during an early period after transplantation (Figure 1).

We then examined the TCRBV repertoire of the CD4 CD28 T cell subset using FACS-sorted cells from three selected patients, and identified TCRBV subfamilies predominantly used by those cells (Table 1). Clonalities of CD4 CD28 T cells carrying the skewed TCRBV subfamilies were determined by CDR3 size spectratyping, and they were compared to those of CD4 CD28 T cells (Figure 2). The CD4 CD28 T cell subset showed less diversity than CD4 CD28 T cells. Thus, oligoclonal expansion was evident in the CD4 CD28 T cell subset after allogeneic hematopoietic cell transplantation (HCT).

Identification of the same clones within both CD4 CD28 and CD4 CD28 T cell subsets

If the clonally expanded CD4 CD28 T lymphocytes are derived from CD4 CD28 T cells, one would expect to

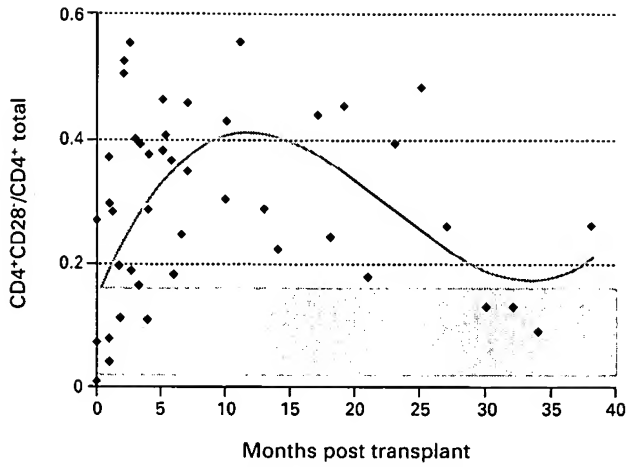


Figure 1 Expansion of the CD28 subset in CD4 T cells after allogeneic bone marrow transplantation. Data from 23 patients receiving allogeneic marrow grafts are graphically presented. A regression curve using a three-dimensional polynomial equation was added. The shaded area shows the mean one standard deviation of the data from eleven healthy individuals.

identify T cells with the same CDR3 sequences within both subsets. We subcloned PCR products of skewed TCR-β chains to see if identical CDR3 sequences could be found in both CD4 CD28 and CD4 CD28 subsets. We found identical sequences in both subsets in all three patients examined (Table 2).

Discussion

We have demonstrated the clonal expansion of CD4 CD28 T lymphocytes after human allogeneic HCT and the presence of identical clonal T cells within both CD4 CD28 and CD4 CD28 T cell subsets. These results suggest that both subsets are phenotypic variants of the same lineage. It remains uncertain whether the transition between the two subsets is unidirectional or bi-directional, although the transition between the CD8 CD28 and the CD8 CD28 T cell subsets appears to be unidirectional. CD8 CD28 T lymphocytes are infrequent in cord blood, thymus and lymph nodes but this subset increases during human aging.[19] CD8 CD28 T cells have been demonstrated to have shorter telomeres than CD8 CD28 T cells.[20] Moreover, it has been recently reported that CD8 CD28 T cells can be generated from chronically stimulated CD8 CD28 T cells.[21] These results support the hypothesis that the CD8 CD28 T cell clones arise from the CD8 CD28 T cell population. It has been reported that CD4 CD28 T cells are found rarely in healthy individuals younger than 40 years and aging is associated with clonal expansion of the CD4 CD28 T cell population.[22,23] By analogy with the study of CD8 CD28 T cells, it is likely that the transition between both CD4 CD28 and CD4 CD28 T cell subsets may be also unidirectional. However, further *in vitro* and *in vivo* experiments are required to address this issue.

Recently, we have also found that clonal expansion of CD8 CD28 T cells is observed during the early period after allogeneic marrow transplantation, and that there are T cell clones with identical CDR3 sequences within both CD8 CD28 and CD8 CD28 T cell subsets.[11] Loss of CD28 expression appears to be associated with clonal predominance of T lymphocytes after allogeneic HCT. Clonal expansion is one of the principal features of T cell immune responses against antigens,[24,25] and there is accumulating evidence for an association between the loss of CD28 expression and T lymphocyte aging.[9,10] CD8 CD28 T cells have recently been considered to be the terminally differentiated effector cells in a state of replicative senescence.[6,9] Accumulation of CD4 CD28 T cells following allogeneic HCT may be explained by several mechanisms. Like CD8 T lymphocytes, CD4 T cells may be chronically stimulated by certain antigens present in the host and then down-regulate the CD28 expression during clonal expansion.[21] Alternatively, CD4 CD28 T cells may be resistant to cell death and have a slow turnover. There is evidence for clonal expansion of CD4 CD28 T cells in rheumatoid arthritis patients, and resistance to apoptosis and elevated expression of Bcl-2 are responsible for clonal expansion of CD4 CD28 T cells.[7,8,26]

The antigen specificity and functional properties of clonally expanded CD4 CD28 T cells found in recipients of allogeneic hematopoietic stem cell grafts are largely

Table 1 Usage of TCRBV in CD4 CD28 and other T cell subsets[a]

Patient No.	*TCRBV probe*	*Target TCRBV segment*	*PBMC*	*% frequency*[b]			
				CD4 CD28	*CD4 CD28*	*CD8 CD28*	*CD8 CD28*
UPN1	VB061	BV6S1	2	2	24	1	6
	VB064	BV6S4	11	11	22	14	8
	VB141	BV14S1	4	2	15	3	5
UPN2	VB041	BV4S1, BV4S2	3	7	20	1	2
	VB131	BV13S1, BV13S8	3	22	27	2	5
UPN3	VB091	BV9S1, BV9S2	2	3	53	1	0
	VB131	BV13S1, BV13S8	15	5	13	7	40
	VB141	BV14S1	4	2	14	4	1

[a]TCRBV subfamilies were selected in each patient based on the usage by the CD4 CD28 cells exceeding 10%. CD4 CD28 T cells accounted for 8.7, 8.1 and 9.5% of total lymphocytes from patients UPN1, UPN2 and UPN3, respectively.
[b]Each value indicates the percentage of T cells bearing the indicated TCRBV in the respective T cell subset.

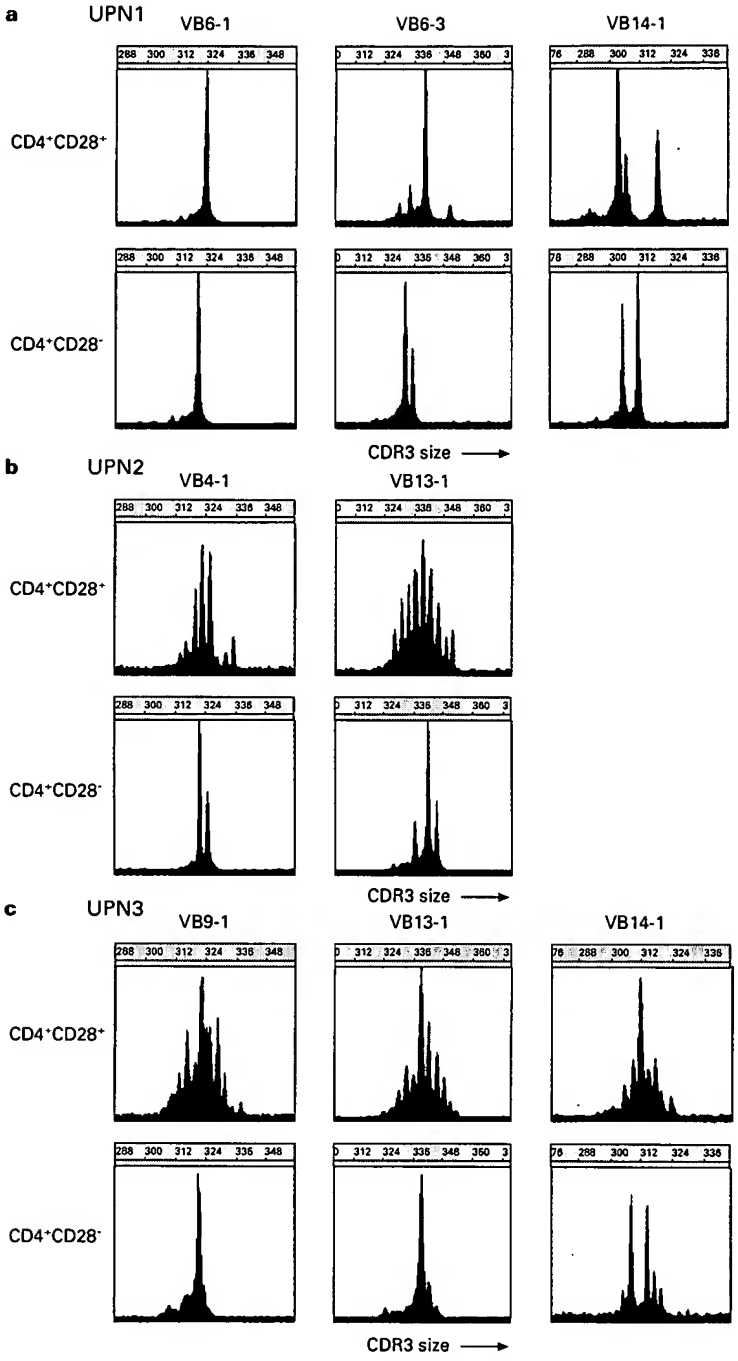


Figure 2 CDR3 size distribution pattern of skewed TCRBV subfamilies in the CD4 CD28 and CD4 CD28 T cell subsets. (a) UPN1, (b) UPN2, (c) UPN3.

unknown. However, there are some clues that allow us to speculate about their specificity and functions. Hooper *et al*[27] have reported that expansion of CD4 CD28 CD57 T cells in rheumatoid arthritis patients are found only in cytomegalovirus (CMV) seropositive donors. The CD8 CD28 T cell population has been shown to contain virus-specific memory cytotoxic T lymphocytes that respond to human CMV and human immunodeficiency virus (HIV).[28–32] It is well known that reactivation of CMV occurs during the first 3 months after allogeneic marrow transplantation and there is a report describing the association between expansion of CD8 T cells and CMV infection.[33] Thus, clonally expanded CD4 CD28 T cells produced following allogeneic HCT may be derived from T cells recognizing antigens that persistently exist in the host such as herpesviruses. This issue can be addressed using major histocompatibility complex (MHC)-peptide tetramers for dominant viral epitopes.[34–36]

Table 2 Deduced amino acid sequences of CDR3 regions of the TCR-β chain in CD4 CD28 and CD4 CD28 T cells

Patient No.	*Cells*	*TCRBV segment*[a]	*TCRBV*	*N–D–N*	*TCRBJ*	*TCRBJ segment*	*Colony frequency*
UPN1	CD28 CD4	BV14S1	LYFCASS	SAWG	TEAFFGQGTRL	BJ1S1	5/6
			LYFCASS	ALGLAGD	PYNEQFFGPGT	BJ2S1	1/6
	CD28 CD4	BV14S1	LYFCASS	SAWG	TEAFFGQGTRL	BJ1S1	6/8
			LYFCASS	SDRGI	NQPQHFGDGTR	BJ1S5	2/8
UPN2	CD28 CD4	BV4S1	IYLCSVE	QGT	NEQFFGPGTRL	BJ2S1	1/8
			IYLSCVE	TGTA	NYGYTFGSGTR	BJ1S2	1/8
			IYLC	GADLSGTSGLLG	EQFFGPGTRLT	BJ2S1	1/8
			IYLCSVE	DQVLP	EAFFGQGTRLT	BJ1S1	2/8
			IYLCSVE	LAGTSGS	YEQYFGPGTRL	BJ2S7	1/8
			IYLCSV	GVTGA	GYTFGSGTRLT	BJ1S2	1/8
			IYLCS	AESY	YEQYFGPGTRL	BJ2S7	1/8
	CD28 CD4	BV4S1	IYLCSVE	DQVLP	EAFFGQGTRLT	BJ1S1	4/7
			IYLCSV	IRQGVNT	EAFFGQGTRLT	BJ1S1	1/7
			IYLCSVE	LGNI	NYGYTFGSGTR	BJ1S2	1/7
			IYLCSV	IRQGVNT	EAFFGQGTRLT	BJ1S1	1/7
UPN3	CD28 CD4	BV13S1	VYFCASS	NSGPD	TDTQYFGPGTR	BJ2S3	1/8
			VYFCASS	LTGTS	NEKLFFGSGTQ	BJ1S4	1/8
			VYFCAS	TGDH	GYTFGSGTRLT	BJ1S2	1/8
			VYFCASSY	FG	SNQPQHFGDGT	BJ1S5	1/8
		BV13S3	VYFCASSEA	LPGTRSG	TQYFGPGTRLL	BJ2S5	1/8
			VYFCASS	PGLAGG	TDTQYFGPGTR	BJ2S3	1/8
			VYFCAS	RRAGL	QETQYFGPGTR	BJ2S5	1/8
		BV13S9	VYFCAS	GRT	YEQYFGPGTRL	BJ2S7	1/8
	CD28 CD4	BV13S1	VYFCASSY	FG	SNQPQHFGDGT	BJ1S5	5/7
		BV13S3	VYFCASS	DGTG	YEQYFGPGTRL	BJ2S7	1/7
		BV13S9	VYFCASSYS	GGSG	YEQYFGPGTRL	BJ2S7	1/7

[a]TCRBV gene segments are described according to the nomenclature reported by Concannon and Robinson.[37]

Acknowledgements

We are grateful to the hematology staff at Akita University Medical Center for their treatment of the patients in this study. This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan (Grant No. 08670508, 10670932), the Yamashita Taro-Kensho Memorial Foundation and the Uehara Memorial Foundation.

References

1 June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. *Immunol Today* 1994; **15**: 321–331.

2 Nociari MM, Telford W, Russo C. Postthymic development of CD28 CD8 T cell subset: age-associated expansion and shift from memory to naive phenotype. *J Immunol* 1999; **162**: 3327–3335.

3 Brinchmann JE, Dobloug JH, Heger BH *et al*. Expression of costimulatory molecule CD28 on T cells in human immunodeficiency virus type 1 infection: functional and clinical correlations. *J Infect Dis* 1994; **169**: 730–738.

4 Lewis DE, Tang DS, Adu-Oppong A *et al*. Anergy and apoptosis in CD8 T cells in HIV-infected persons. *J Immunol* 1994; **153**: 412–420.

5 Leroy E, Calvo CF, Divine M *et al*. Persistence of T8 /HNK-1 suppressor lymphocytes in the blood of long-term surviving patients after bone marrow transplantation. *J Immunol* 1986; **137**: 2180–2189.

6 Hamann D, Roos MT, van Lier RA. Faces and phases of human CD8 T-cell development. *Immunol Today* 1999; **20**: 177–180.

7 Schmitd D, Goronzy JJ, Weyand CM. CD4 CD7 CD28 T cells are expanded in rheumatoid arthritis and are characterized by autoreactivity. *J Clin Invest* 1996; **97**: 2027–2037.

8 Imberti L, Sottini A, Signorini S *et al*. Oligoclonal CD4 CD57 T-cell expansions contribute to the imbalanced T-cell receptor repertoire of rheumatoid arthritis patients. *Blood* 1997; **89**: 2822–2832.

9 Effros RB, Pawelec G. Replicative senescence of T cells: does the Hayflick Limit lead to immune exhaustion? *Immunol Today* 1997; **18**: 450–454.

10 Globerson A, Effros RB. Ageing of lymphocytes and lymphocytes in the aged. *Immunol Today* 2000; **21**: 515–521.

11 Horiuchi T, Hirokawa M, Kawabaya Y *et al*. Identification of the T-cell clones expanding within both CD8 CD28 and CD8 CD28 T-cell subsets in recipients of allogeneic hematopoietic cell grafts and its implication in post-transplant skewing of T cell receptor repertoire. *Bone Marrow Transplant* (in press).

12 Storb R, Deeg HJ, Whitehead J *et al*. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *New Engl J Med* 1986; **314**: 729–735.

13 Matsutani T, Yoshioka T, Tsuruta Y *et al*. Analysis of TCRAV and TCRBV repertoires in healthy individuals by microplate hybridization assay. *Hum Immunol* 1997; **56**: 57–69.

14 Matsutani T, Yoshioka T, Tsuruta Y *et al*. Restricted usage of TCRAV and TCRBV repertoires after human allogeneic hematopoietic transplantation. *Br J Haematol* 2000; **109**: 759–769.

15 Pannetier C, Cochet M, Darche S *et al*. The sizes of the CDR3 hypervariable regions of the murine T-cell receptor beta chains vary as a function of the recombined germ-line segments. *Proc Natl Acad Sci USA* 1993; **90**: 4319–4323.

16 Hirokawa M, Horiuchi T, Kitabayashi A *et al*. Delayed recov-

ery of CDR3 complexity of the T cell receptor β chain in recipients of allogeneic bone marrow transplants who had virus-associated interstitial pneumonia: monitor of T-cell function by CDR3 spectratyping. *J Allergy Clin Immunol* 2000; **106**: S32–39.

17 Yoshioka T, Matsutani T, Iwagami S *et al.* Quantitative analysis of the usage of human T cell receptor alpha and beta chain variable regions by reverse dot blot hybridization. *J Immunol Meth* 1997; **201**: 145–155.

18 Hirokawa M, Horiuchi T, Kawabata Y *et al.* Reconstitution of γδ T cell repertoire diversity after human allogeneic hematopoietic cell transplantation and the role for peripheral expansion of mature T cell population in the graft. *Bone Marrow Transplant* 2000; **26**: 177–185.

19 Posnett DN, Edinger JW, Manavalan JS *et al.* Differentiation of human CD8 T cells: implications for *in vivo* persistence of CD8 CD28 cytotoxic effector clones. *Int Immunol* 1999; **11**: 229–241.

20 Monteiro J, Batliwalla F, Ostrer H, Gregersen PK. Shortened telomeres in clonally expanded CD28 CD8 T cells imply a replicative history that is distinct from their CD28 CD8 counterparts. *J Immunol* 1996; **156**: 3587–3590.

21 Fiorentini S, Malacarne F, Ricotta D *et al.* Generation of CD28 cells from long-term-stimulated CD8 CD28 T cells: a possible mechanism accounting for the increased number of CD8 CD28 T cells in HIV-1-infected patients. *J Leukoc Biol* 1999; **65**: 641–648.

22 Colombatti A, Doliana R, Schiappacassi M *et al.* Age-related persistent clonal expansions of CD28( ) cells: phenotypic and molecular TCR analysis reveals both CD4( ) and CD4( )CD8( ) cells with identical CDR3 sequences. *Clin Immunol Immunopathol* 1998; **89**: 61–70.

23 Weyand CM, Brandes JC, Schmidt D *et al.* Functional properties of CD4 CD28 T cells in the aging immune system. *Mech Ageing Dev* 1998; **102**: 131–147.

24 McHeyzer-Williams MG, Davis MM. Antigen-specific development of primary and memory T cells *in vivo*. *Science* 1995; **268**: 106–111.

25 Maini MK, Casorati G, Dellabona P *et al.* T-cell clonality in immune responses. *Immunol Today* 1999; **20**: 262–266.

26 Schirmer M, Vallejo AN, Weyand CM, Goronzy JJ. Resistance to apoptosis and elevated expression of bcl-2 in clonally expanded CD4 CD28 T cells from rheumatoid arthritis patients. *J Immunol* 1998; **161**: 1018–1025.

27 Hooper M, Kallasm EG, Coffin D *et al.* Cytomegalovirus seropositivity is associated with the expansion of CD4 CD28 and CD8 CD28 T cells in rheumatoid arthritis. *J Rheumatol* 1999; **26**: 1452–1457.

28 Wang ECY, Moss PAH, Frodsham P *et al.* CD8highCD57 T lymphocytes in normal, healthy individuals are oligoclonal and respond to human cytomegalovirus. *J Immunol* 1995; **155**: 5046–5056.

29 Weekes MP, Carmichael AJ, Wills MR *et al.* Human CD28 CD8 T cells contain greatly expanded functional virus-specific memory CTL clones. *J Immunol* 1999; **162**: 7569–7577.

30 Lewis DE, Yang L, Luo W *et al.* HIV-specific cytotoxic T lymphocyte precursors exist in a CD28 CD8 T cell subset and increase with loss of CD4 T cells. *AIDS* 1999; **13**: 1029–1033.

31 Dalod M, Sinet M, Deschemin JC *et al.* Altered *ex vivo* balance between CD28 and CD28 cells within HIV-specific CD8 T cells of HIV-seropositive patients. *Eur J Immunol* 1999; **29**: 38–44.

32 Caruso A, Licenziati S, Canaris AD *et al.* Contribution of CD4 , CD8 CD28 , and CD8 CD28 T cells to CD3 lymphocyte homeostasis during the natural course of HIV-1 infection. *J Clin Invest* 1998; **101**: 137–144.

33 Dolstra H, de Wiel-van Kemenade EV, Witte TD, Preijers F. Clonal predominance of cytomegalovirus-specific CD8 cytotoxic T lymphocytes in bone marrow recipients. *Bone Marrow Transplant* 1996; **18**: 339–345.

34 Altman JD, Moss PAH, Goulder PJR *et al.* Phenotypic analysis of antigen-specific T lymphocytes. *Science* 1996; **274**: 94–96.

35 Engstrand M, Tournay C, Peyrat MA *et al.* Characterization of CMVpp65-specific CD8 T lymphocytes using MHC tetramers in kidney transplant patients and healthy participants. *Transplantation* 2000; **69**: 2243–2250.

36 Kwok WW, Liu AW, Novak EJ *et al.* HLA-DQ tetramers identify epitope-specific T cells in peripheral blood of herpes simplex virus type 2-infected individuals: direct detection of immunodominant antigen-responsive cells. *J Immunol* 2000; **164**: 4244–4249.

37 Concannon P, Robinson MA. Human T-cell receptor gene nomenclature. *Ann NY Acad Sci* 1995; **756**: 124–129.

A6

- Japanese Journal of Clinical Oncology
- Volume 28, Number 12
- Pp. 723-728

Expression of [alpha]-Fetoprotein and Prostate-specific Antigen Genes in Several Tissues and Detection of mRNAs in Normal Circulating Blood by Reverse Transcriptase-Polymerase Chain Reaction

Tomoyoshi Ishikawa[1], Hironobu Kashiwagi[1], Yoko Iwakami[1], Misako Hirai[1], Tomonori Kawamura[1], Yuji Aiyoshi[2], Toru Yashiro[2], Yoshihiro Ami[3], Kazuhiko Uchida[1] and Masanao Miwa[1]

[1]Department of Biochemistry and Molecular Oncology, Institute of Basic Medical Sciences and Center for Tsukuba Advanced Research Alliance, [2]Department of Surgery, Institute of Clinical Medicine, University of Tsukuba and [3]Department of Urology, Tsukuba Gakuen Hospital, Tsukuba, Ibaraki, Japan

Background: **[alpha]-Fetoprotein (AFP) and prostate-specific antigen (PSA) in serum are widely used as tumor markers in the evaluation of prognosis and management of patients with hepatocellular carcinoma and prostate cancer, respectively. To establish the molecular diagnosis of cancer, reverse transcriptase polymerase chain reaction (RT-PCR) for *AFP* and *PSA* was used to identify circulating cancer cells in the blood of cancer patients. Here, we examined the tissue-specificity of *AFP* and *PSA* and tested whether *AFP* and *PSA* are suitable targets in the detection of certain cancer cells by RT-PCR using peripheral blood samples.**

Methods: **Tissue specificity of *AFP* and *PSA* was analyzed by Northern blotting and RT-PCR. Probes for *AFP* and *PSA* were hybridized with poly A^+ RNAs from 50 human tissues. RT-PCR for *AFP* and *PSA* mRNA was performed using several cancerous tissues and normal tissues and peripheral blood cells from seven healthy volunteers.**

Results: **Broad expression of *AFP* was observed in several tissues and a large amount of *AFP* mRNA was found in fetal liver. *PSA* was expressed in prostate, salivary gland, pancreas and uterus. By RT-PCR, *AFP* and *PSA* mRNA were detected in several tumors, including salivary pleomorphic adenoma, hilar bile duct carcinoma, pancreatic carcinoma, transitional cell carcinoma of urinary bladder and thyroid papillary carcinoma. Furthermore, *AFP* and *PSA* mRNAs were frequently detected by RT-PCR, even in peripheral blood cells from healthy volunteers.**

Conclusions: **Neither *AFP* nor *PSA* showed tissue-specific expression. *AFP* and *PSA* mRNA were detected in several diseased and non-diseased tissues and normal circulating blood by RT-PCR.**

Key words: tissue-specific expression - [alpha]-fetoprotein - prostate-specific antigen - reverse transcriptase polymerase chain reaction - molecular diagnosis

INTRODUCTION

In Japan, about 300 000 people die yearly of cancer, making this disease the leading cause of death in adults (1). Non-invasive and highly sensitive diagnostic methods against cancer will undoubtedly have a major impact on cancer diagnosis and therapy. [alpha]-Fetoprotein (AFP) and prostate-specific antigen (PSA) in serum are valuable biological markers for the diagnosis, prognosis and management of patients with hepatocellular carcinoma (HCC) and prostate cancer, respectively (2,3). AFP is a serum protein mainly synthesized in mammalian fetal liver cells (4-8). PSA is a glycoprotein (9,10) up-regulated by androgenic hormones at the transcriptional level (11) and mainly produced in the prostate. Recently, the detection of circulating cancer cells by reverse transcriptase-polymerase chain reaction (RT-PCR) for the evaluation of post-operative prognosis, molecular staging of cancers (12) and diagnosis of micro-metastasis (13) has been reported. In these reports, tumor-specific mRNA has been employed to detect circulating cancer cells in peripheral blood cells of cancer patients. Detection of *AFP* and *PSA* mRNA by RT-PCR in peripheral blood has become one of the most useful molecular biomarkers (12,14-20) in cancer diagnosis. PCR is an extremely sensitive method to detect even small amounts of DNA/RNA sequences. Because of its high sensitivity, false-positive results can possibly occur even if *AFP* and *PSA* mRNA are used as targets for RT-PCR. Recent studies have reported detection of *PSA* mRNA by RT-PCR in several cancers other than prostate cancer (21-25). Expression of *AFP* in normal renal cells has also been detected (26). Low levels of *PSA* and *AFP* transcripts in peripheral blood from non-cancer patients can possibly affect the ability of RT-PCR to detect circulating cancer cells. Tissue specificity of gene expression of *PSA* and *AFP* is crucial in the establishment of cancer diagnosis by RT-PCR using peripheral blood.

In this study, we analyzed the tissue specificity of *AFP* and *PSA* in 43 human adult and seven fetal tissues by Northern blotting and examined the expression of *AFP* and *PSA* by RT-PCR using several tumors and normal peripheral blood cells.

MATERIALS AND METHODS

Probes

cDNA probes for *AFP* and *PSA* mRNAs were prepared by RT-PCR using total RNA from normal liver and prostate, respectively. The sequences of *AFP* primers were 5-GTT GCC AAC TCA GTG AGG AC-3 for the forward primer (AFP-F) and 5-GAG CTT GGC ACA GAT CCT TA-3 for the reverse primer (AFP-R). The sequences of *PSA* primers were 5-CCC ACA CCC GCT CTA CGA TA-3 for the forward primer (PSA-F) and 5-ACC TTC TGA GGG TGA ACT TGC G-3 for the reverse primer (PSA-R). The PCR products of *AFP* and *PSA* cDNAs are 240 and 289 base pairs in length, respectively. The RT-PCR products of *AFP* and *PSA* were purified with a MERmaid Kit (BIO 101, Vista, CA) and subcloned into pCR 2.1 (Original TA Cloning Kit, Invitrogen, San Diego, CA). The resulting plasmids were digested by *Eco*RI and the inserts were electrophoretically separated on a 0.8% agarose gel for purification. Human *ubiquitin* cDNA (CLONTECH, Palo Alto, CA) was used for standardization. The isolated DNA fragments were radiolabeled with [[alpha]-^{32}P]dCTP using a Multiprime DNA Labeling System (Amersham, UK). Plasmid inserts were sequenced by the dye primer method using a DNA

Sequencing Kit with Dye Primer Cycle Sequencing Ready Reaction (Perkin-Elmer-Cetus, Foster City, CA) with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer-Cetus).

Northern Blotting

Tissue specificity was analyzed by using a Human RNA Master Blotting (CLONTECH). Hybridization was performed according to the manufacturer's protocol. Amounts of 20 ng of probes were labeled with [[alpha]-^{32}P]dCTP and then added to a mixture of 25 μg of human Cot-1 DNA (GIBCO BRL, Rockville, MD), 125 μg of sheared salmon testis DNA and 5 ml of ExpressHyb solution (CLONTECH). The blot was incubated at 65°C for 16 h. After washing four times with 2× SSC and 1% SDS at 65°C for 20 min. two additional 20 min washes were performed in 0.1× SSC and 0.5% SDS at 65°C. The damp blot was exposed on an Imaging Plate (FujiPhoto Film, Tokyo). Dots on the autoradiograph were analyzed and quantified with a BAS 5000 Imaging Analyzer (FujiPhoto Film). To determine the tissue specificity of the gene expression, the intensity percentage (% intensity) of each dot was calculated as follows according to the manufacturer's protocol. The raw data of the position of yeast transfer RNA on the blot (H2) was set as the background of the blot (data not shown in Figs 1 and 2). Signal intensity values for each dot were divided by the product of the original mRNA amounts (ng) and the scan area (mm^2). The value thus obtained in certain tissues was divided by the sum of all obtained values of the blotting and shown as % intensity. The ratio of the expressed mRNA in each tissue was obtained from this calculation.

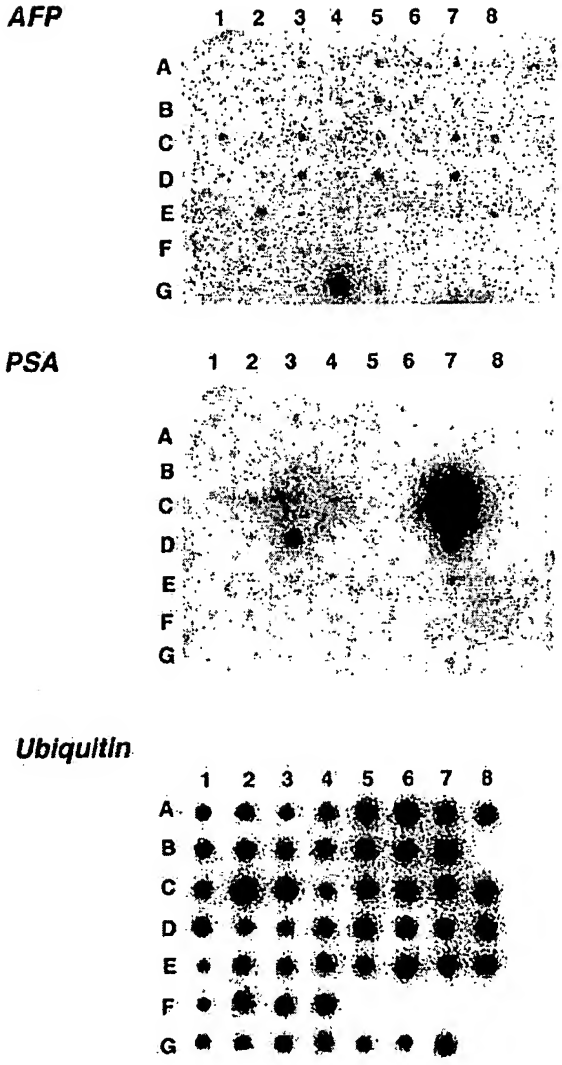


Figure 1. Expression of *AFP* and *PSA* genes in various tissues. *AFP* and *PSA* cDNAs were hybridized with poly A^+ RNAs from 50 tissues. Hybridization of *ubiquitin* cDNA was performed for standardization. *AFP* was expressed in more than 30 types of tissue. A strong signal was observed in fetal liver. *PSA* was expressed mainly in prostate, salivary gland and pancreas. The type and position of poly A^+ RNAs on the blot are as follows: A1, whole brain; A2, amygdala; A3, caudate nucleus; A4, cerebellum; A5, cerebral cortex; A6, frontal lobe; A7, hipocampus; A8, medulla oblongata; B1, occipital lobe; B2, putamen; B3, substantia nigra; B4, temporal lobe; B5, thalamus; B6, subthalamic nucleus; B7, spinal cord; C1, heart; C2, aorta; C3, skeletal muscle; C4, colon; C5, bladder; C6, uterus; C7, prostate; C8, stomach; D1, testis; D2, ovary; D3, pancreas; D4, pituitary gland; D5, adrenal gland; D6, thyroid gland; D7, salivary gland; D8, mammary gland; E1, kidney; E2, liver; E3, small intestine; E4, spleen; E5, thymus; E6, peripheral lymphocytes; E7, lymph node; E8, bone marrow; F1, appendix; F2, lung; F3, trachea; F4, placenta; G1, fetal brain; G2, fetal heart; G3, fetal kidney; G4, fetal liver; G5, fetal spleen; G6, fetal thymus; and G7, fetal lung.

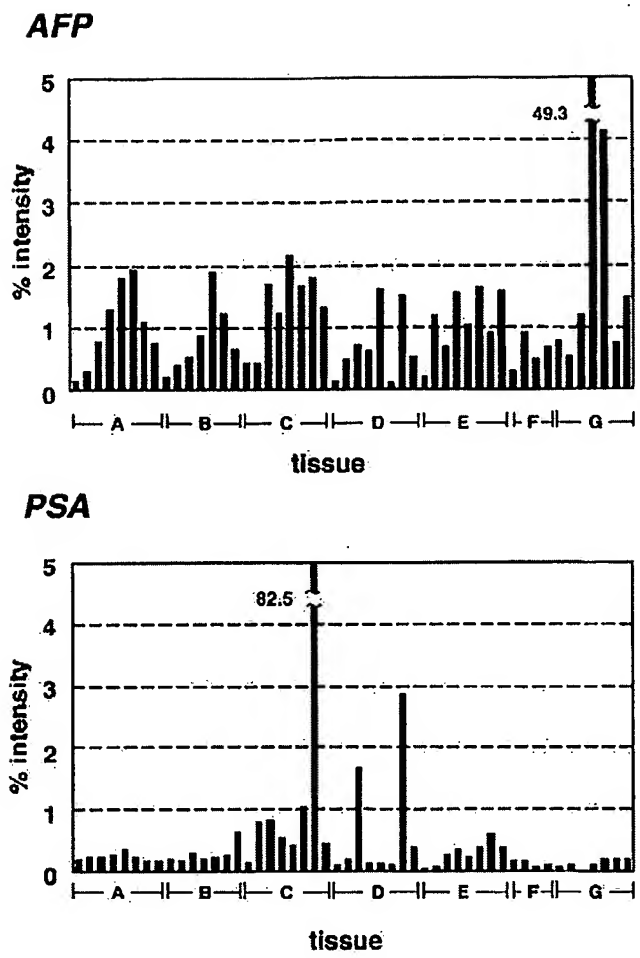


Figure 2. Intensity percentage of *AFP* and *PSA* gene expression. Each bolt was analyzed using Phosphorimager and quantified with the BAS 5000 Imaging Analyzer. The % intensity for each tissue was calculated as described in Materials and Methods. Tissue samples are indicated in groups from A to G. From the left, A1-8, B1-7, C1-8, D1-8, E1-8, F1-4 and G1-7 are shown. Definitions as in Fig. 1.

RNA Preparation and RT-PCR

Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction (AGPC) method (27) from the following tissues: normal liver, hilar bile duct carcinoma, prostate cancer, benign prostatic hypertrophy, normal prostate, pancreatic carcinoma, normal pancreas, pleomorphic adenoma of parotid gland, normal parotid gland, bladder cancer, thyroid papillary carcinoma and normal thyroid gland. Whole blood was subjected to a Ficcoll-Conray gradient (IBL, Fujioka) and the nucleate cells were collected. Total RNAs from 5 ml of peripheral whole

blood of seven healthy volunteers were also extracted by the AGPC method. Total RNA was finally dissolved in diethylpyrocarbonate-treated water to a final concentration of 1 μg/μl. Prior to reverse transcription, the total RNA solution was heated at 70°C for 10 min and then immediately placed on ice for 5 min. cDNA was synthesized using a RNA PCR Core Kit (Perkin-Elmer-Cetus) from 1 μg of total RNA in 20 μl of reaction mixture containing 1× PCR Buffer II, 1 mM dNTP, 2.5 mM oligo-d$(T)_{16}$, 20 units of RNasin and 50 units of murine leukemia virus (MuLV) reverse transcriptase. Reverse transcription was carried out at 42°C for 15 min. A 5 μl volume of the reaction mixture was used for PCR. PCR was carried out in 50 μl of reaction mixture containing 1× PCR Buffer II containing AmpliTaq Gold (Perkin-Elmer-Cetus) DNA polymerase. To obtain the most effective amplification by AmpliTaq Gold DNA polymerase, the PCR cycle was increased up to 50 cycles according to the manufacturer's protocol. PCR cycles for *AFP* and *PSA* were as follows: initial denaturation at 95°C for 10 min, followed by 50 cycles of 94°C for 1 min, 58°C for 2 min, 72°C for 3 min and final extension at 72°C for 7 min. RT-PCR of [beta]-*actin* was used as an internal control as described (28). The sequences of [beta]-*actin* primers are 5-AGA GAT GGC CAC GGC TGC TT-3 for the forward primer in exon 4 and 5-ATT TGC GGT GGA CGA TGG AG-3 for the reverse primer in exon 6. The PCR product is 406 bp in length. After reverse transcription, amplification was performed with 0.2 U of AmpliTaq DNA polymerase (Perkin-Elmer-Cetus) in 2.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% gelatin (w/v), 0.25 mM of each dNTP and 5 pmol of each primer, by a precycle at 94°C for 3 min and subsequently 40 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C in a thermal cycler (Perkin-Elmer-Cetus). RT-PCR products of *AFP*, *PSA* and [beta]-*actin* were separated electrophoretically on a 2.0% agarose gel and detected with ethidium bromide staining.

RESULTS

Tissue Specificity of AFP and PSA Gene Expression

Fig. 1 shows the expression of *AFP* and *PSA* genes in 50 human tissues including fetal organs. The total quantity of mRNA in each dot ranges between 95 (E6; peripheral leukocytes) and 461 ng/dot (D3; pancreas). The *AFP* gene was highly expressed in fetal liver. However, the broad expression pattern of the *AFP* gene was observed. Positive signals were found in several kinds of tissue, including peripheral leukocytes. *AFP* mRNA was also detected in various kinds of fetal tissues. Fig. 2 shows the % intensity of expression of *AFP* and *PSA* genes to evaluate the tissue specificity of the gene expression. A high % intensity was observed for *AFP* mRNA in fetal liver (49.3%), fetal spleen (4.2%), bladder (2.1%), frontal lobe (1.9%) and thalamus (1.9%). Peripheral leukocytes showed expression of *AFP* at 1.64% intensity. Thus, *AFP* mRNA was detected in almost all tissues, including peripheral leukocytes, even at low levels.

The *PSA* gene also showed broad expression patterns as shown in Figs 1 and 2. Strong signals were observed in prostate, salivary gland, pancreas and uterus. The % intensities of *PSA* were prostate 82.5%, salivary gland 2.9%, pancreas 1.7% and uterus 1.0%. Signals for *PSA* were detected in almost all tissues, including peripheral leukocytes (0.4%). Small amounts of *PSA* mRNA were also found in various kinds of adult tissues. Fetal kidney was the only tissue where the signal for *PSA* mRNA was not detected.

Detection of *AFP* and *PSA* mRNA by RT-PCR in Cancers and Normal Peripheral Blood

We analyzed *AFP* and *PSA* expression in several cancer tissues to examine the cancer specificity of their expression. *AFP* mRNA was detected in normal liver, hilar bile duct carcinoma, prostate cancer, benign prostatic hypertrophy, normal prostate, pancreatic carcinoma, normal pancreas, pleomorphic adenoma of parotid gland, normal parotid gland, bladder cancer, thyroid papillary carcinoma and normal thyroid gland (Fig. 3). *PSA* mRNA was detected not only in prostate but also in normal liver, hilar bile duct carcinoma, pancreas carcinoma, normal pancreas, pleomorphic adenoma of parotid gland, normal parotid gland, bladder cancer, thyroid papillary carcinoma and normal thyroid gland (Fig. 3). By RT-PCR, *AFP* mRNA was detected in peripheral blood from six out of seven healthy volunteers and *PSA* mRNA was also detected in five out of seven healthy volunteers (Fig. 4). [beta]-*actin* mRNA as internal control was detected in all cases by RT-PCR. In some cases, contaminated genomic DNA was observed at about 432 bp in PCR for *PSA*, whereas no band from genomic DNA was observed in PCR for *AFP* because of the large size ([sim]1600 bp) of the expected PCR product from genomic DNA and the small amount of contaminated genomic DNA.

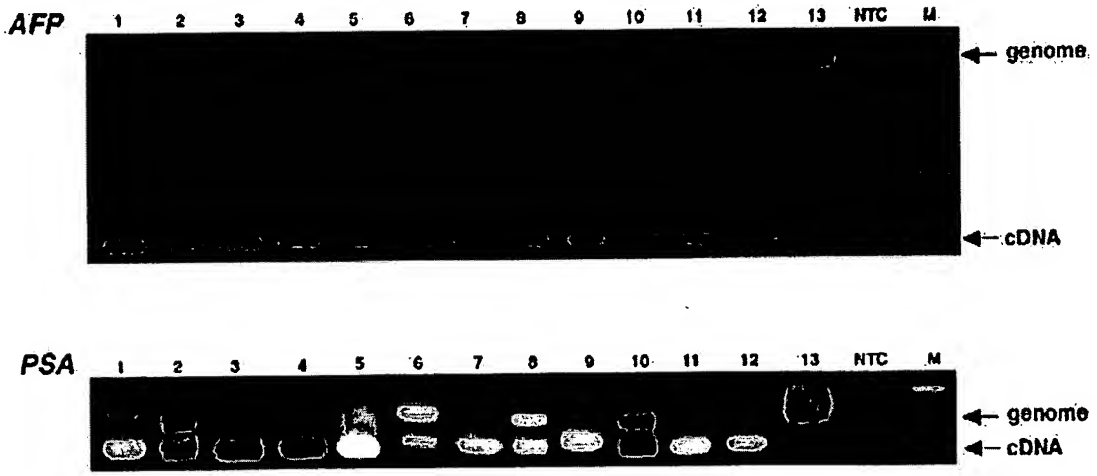


Figure 3. Detection of *AFP* and *PSA* mRNA in various cancers by RT-PCR. *AFP* and *PSA* transcripts were 240 and 289 bp, respectively. Lanes in each panel are as follows: 1, normal liver; 2, hilar bile duct carcinoma; 3, prostatic cancer; 4, benign prostatic hypertrophy; 5, normal prostate; 6, pancreatic carcinoma; 7, normal pancreas; 8, pleomorphic adenoma of parotid gland; 9, normal parotid gland; 10, bladder cancer; 11, thyroid papillary carcinoma; 12, normal thyroid gland; 13, DNA from normal lymphocytes as a negative control; NTC, no template control; M, DNA marker (pBR322 *Hin*fI digest).

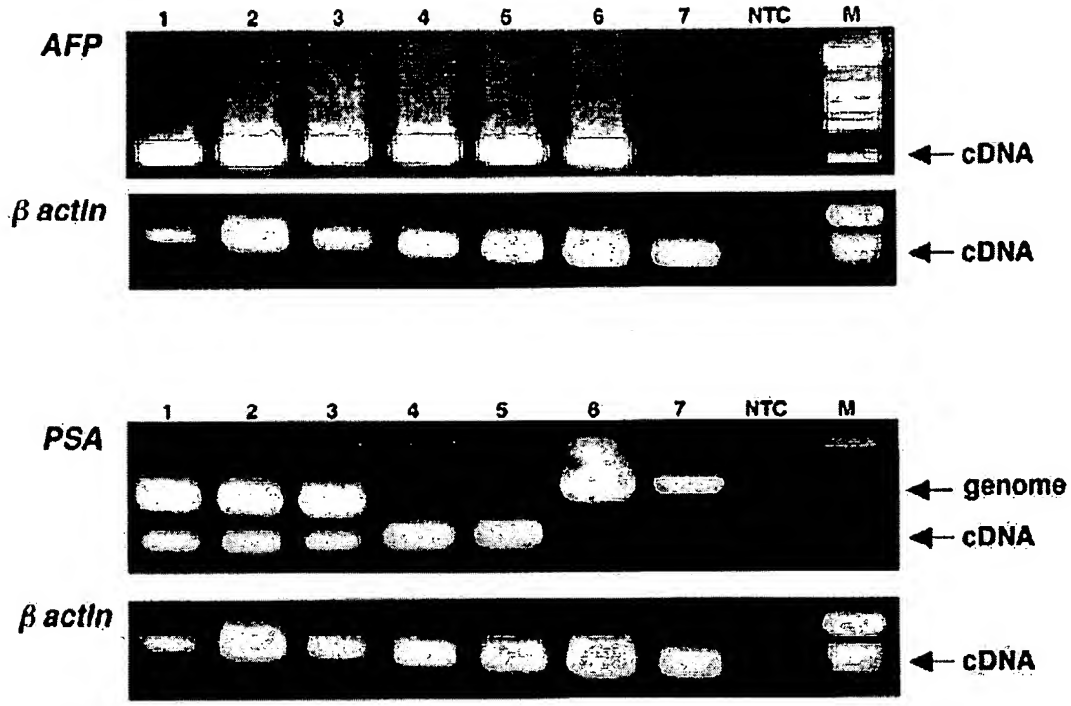


Figure 4. Detection of *AFP* and *PSA* mRNA in peripheral blood cells from healthy volunteers by RT-PCR. Lanes 1-7, peripheral blood cells from healthy volunteers; NTC, no template control; M, DNA marker (pBR322 *Hin*fI digest).

DISCUSSION

We have found that various human tissues, including peripheral leukocytes, expressed the *AFP* and *PSA* genes. mRNAs for these genes were frequently detected in normal circulating blood. Northern blot analysis using poly A^+ RNAs from 50 human tissues including fetal organs revealed that both *AFP* and *PSA* expression were not tissue specific. Various tissues, including peripheral blood, showed positive spots on the blot. Furthermore, RT-PCR for *AFP* and *PSA* mRNAs indicated the presence of these mRNAs in normal peripheral blood cells. These data suggest a limitation of PCR-based methods with these weak tumor- or tissue-specific mRNAs as targets. Because of the very high sensitivity of PCR, low levels of these tumor-specific transcripts, as shown by Northern blotting (Figs 1 and 2), can be detected in peripheral blood cells from non-cancer patients and healthy volunteers.

By quantification of the signals on the Northern blot, several tissues expressed *AFP* at about 1/25th of the level of the fetal liver. By RT-PCR, we found *AFP* mRNA in cancers and benign

tumors such as adenoma of parotid gland, bile duct cancer, pancreas cancer, bladder cancer and thyroid cancer. *AFP* gene expression in neonatal rat kidney has been reported (26). We also showed *AFP* expression in fetal kidney.

PSA is believed to be expressed exclusively in prostatic epithelial cells. However, in the present study, expression of the *PSA* gene was observed not only in the prostate but also in various other tissues including pancreas, salivary gland and uterus as well as peripheral blood. However, the expression levels in these tissues, excluding prostate, were very low. The % intensity for peripheral leukocytes was 0.4%, whereas prostate showed 82.5% intensity. Even at these low expression levels, we detected *PSA* mRNA in normal tissues such as liver, pancreas, parotid gland and thyroid gland and also in several cancers by RT-PCR (Figs 3 and 4). *PSA* mRNA was frequently detected in peripheral blood cells from healthy volunteers by RT-PCR. These data coincide with previous reports indicating expression of *PSA* in non-prostate cells including normal blood cells (29-32). Convincing evidence has been described for expression of *PSA* in normal tissues such as salivary gland (31), lung (33) and endometrium (34) and also in tumors such as lung cancer (23,35), breast cancer (21), ovarian tumor (24) and other tumors (25). Recent RT-PCR studies suggested expression of the *PSA* gene in breast and lung cancers (21-23,35).

The present study clearly showed the broad expression pattern of the *AFP* and *PSA* genes and no specificity for certain cancers. Detection of the circulating cancer cells by RT-PCR for cancer- and/or tissue-specific mRNA using peripheral blood from patients would be a powerful and non-invasive diagnostic method. The reliability of this method is based on the balance of sensitivity in detection and specificity for cancer. Results for the detection of *PSA* and *AFP* in peripheral blood might be reliable if the sensitivity of detection is reduced. However, the possibility of false-positive results in the highly sensitive RT-PCR must be taken into consideration. The establishment of a molecular diagnostic system to detect circulating cancer cells using RT-PCR for more tumor- and tissue-specific mRNA would have an impact on clinical cancer research.

Acknowledgments

This work was supported in part by Grants-in-Aid for Cancer Research from the Ministry of Education, Sciences, Sports and Culture and from the second term Comprehensive 10-year Strategy for Cancer Control and Cancer Research from the Ministry of Health and Welfare of Japan. We thank Drs T. Todoroki, E. Ueno and H. Hara for collecting samples and for helpful discussions and Ms A. Kikukawa for helping with DNA sequencing.

References

1. Health and Welfare Statistics Association. *Kouseinoshihyou; Kokumineisei-no-Doukou* 1997;44:48-61 (in Japanese).

2. Oesterling JE. Prostate specific antigen: a critical assessment of the most useful marker for adenocarcinoma of the prostate. *J Urol* 1991;145:907-23. MEDLINE Abstract

3. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, Redwine E. Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. *N Engl J Med* 1987;317:909-16. MEDLINE Abstract

4. Kirsch JA, Wise RW, Oilver IT. Post-albumin, a fetal-specific rat plasma protein. Purification, physicochemical and immunological studies. *Biochem J* 1967;102:763-6.

5. Ruoslahti E, Seppala M, Pikko M, Vuopio R. Studies of carcino-fetal proteins. II. Biochemical comparison of [alpha]-fetoprotein from human fetuses and patients with hepatocellular cancer. *Int. J. Cancer* 1971;8:283-8. MEDLINE Abstract

6. Kahan B, Levine L. The occurrence of a serum fetal [alpha]$_1$ protein in developing mice and murine hepatomas and teratomas. *Cancer Res* 1971;31:930-6. MEDLINE Abstract

7. Branch WR, Wild AE. Localization and synthesis of [alpha]-fetoprotein in the rabbit. *Z Zellforsch Mikrosk Anat* 1972;135:501-16. MEDLINE Abstract

8. Tamaoki T, Thomas K, Schindler I. Cell-free studies of developmental changes in synthesis of [alpha]-fetoprotein and albumin in the mouse liver. *Nature* 1974;249:269-71. MEDLINE Abstract

9. Wang MC, Papsidero LD, Kuriyama M, Valenzuela LA, Murphy GP, Chu TM. prostate antigen:a new potential marker for prostatic cancer. *Prostate* 1981;2:89-96. MEDLINE Abstract

10. Watt KWK, Lee PJ, M'Timkulu T, Chan WP, Loor R. Human prostate-specific antigen: structural and functional similarity with serine proteases. *Proc Natl Acad Sci USA* 1986;83:3166-70.

11. Henttu P, Liao S, Vihko P. Androgens up-regulate the human prostate-specific antigen messenger ribonucleic acid (mRNA), but down-regulate the prostatic acid phosphatase mRNA in the LNCaP cell line. *Endocrinology* 1992;130:766-72. MEDLINE Abstract

12. Katz AE, Seaman ES, Olsson CA, O'Toole KM, Raffo AJ, McMahon D, et al. Molecular staging of prostate cancer with the use of an enhanced reverse transcriptase-PCR assay. *Urology* 1994;43:765-75. MEDLINE Abstract

13. Mori M, Mimori K, Ueo H, Tsuji K, Shiraishi T, Barnard GF, et al. Clinical significance of molecular detection of carcinoma cells in lymph nodes and peripheral blood by reverse transcription-polymerase chain reaction in patients with gastrointestinal or breast carcinomas. *J Clin Oncol* 1998;16:128-32. MEDLINE Abstract

14. Funaki N, Tanaka J, Seto S, Kasamatsu T, Kaido T, Imamura M. Highly-sensitive identification of [alpha]-fetoprotein mRNA in circulating peripheral blood of hepatocellular carcinoma patients. *Life Sci* 1995;57:1621-31. MEDLINE Abstract

15. Louha M, Poussin K, Ganne N, Zylberberg H, Nalpas B, Nicolet J, et al. Spontaneous and iatrogenic spreading of liver-derived cells into peripheral blood of patients with primary liver cancer. *Hepatology* 1997;26:998-1005. MEDLINE Abstract

16. Kawakami M, Okaneya T, Furihata K, Nishizawa O, Katsuyama T. Detection of prostate cancer cells circulating in peripheral blood by reverse transcription-PCR for *hKLK2*. *Cancer Res* 1997;57:4167-70. MEDLINE Abstract

17. Seiden MV, Kantoff PW, Krithivas K, Propert K, Bryant M, Haltom E, et al. Detection of circulating tumor cells in men with localized prostate cancer. *J Clin Oncol* 1994;12:2634-9. MEDLINE Abstract

18. Israeli RS, Miller WH, Su SL, Powell CT, Fair WR, Samadi DS, et al. Sensitive nested reverse transcriptase polymerase chain reaction detection of circulating prostate tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. *Cancer Res* 1994;54:6306-10. MEDLINE Abstract

19. Ennis RD, Katz AE, de Vries GM, Heitjan DF, O'Toole KM, Rubin M, et al. Detection of circulating prostate carcinoma cells via an enhanced reverse transcriptase chain reaction assay in patients with early stage prostate carcinoma. Independence from other pretreatment characteristics. *Cancer* 1997;79:2402-8. MEDLINE Abstract

20. Johnson PWM, Burchill SA, Selby PJ. The molecular detection of circulating tumour cells. *Br J Cancer* 1995;72:268-76.

21. Yu H, Giai M, Diamandis EP, Katsaros D, Sutherland DJA, Levesque MA, et al. Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer. *Cancer Res* 1995;55:2104-10. MEDLINE Abstract

22. Lehrer S, Terk M, Piccoli SP, Song HK, Lavagnini P, Luderer AA. Reverse transcriptase chain reaction for prostate-specific antigen may be a prognostic indicator in breast cancer. *Br J Cancer* 1996;74:871-3. MEDLINE Abstract

23. Bilgrami S, Singh NT, Shafi N, Ciesielski T. Raised prostate-specific antigen in adenocarcinoma of lung. *Lancet* 1994;344:1371-2. MEDLINE Abstract

24. Yu H, Diamandis EP, Levesque MA, Asa S, Monne M, Croce CM. Expression of the prostate-specific antigen gene by a primary ovarian carcinoma. *Cancer Res* 1995;55:1603-6. MEDLINE Abstract

25. Levesque M, Yu H, D'Costa M, Diamandis EP. Prostate specific antigen expression by various tumors. *J Clin Lab Anal* 1995;9:123-8. MEDLINE Abstract

26. Gilbert JC, Chiu J-F. Tissue specific control of [alpha]-fetoprotein gene expression. *Biochem Biophys Res Commun* 1984;120:677-85.

27. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9. MEDLINE Abstract

28. Hayakawa S, Saito S, Nemoto N, Chishima F, Akiyama K, Shiraishi H, et al. Expression of recombinase-activating genes (RAG-1 and 2) in human decidual mononuclear cells. *J Immunol* 1994;153:4934-9. MEDLINE Abstract

29. Kamoshida S, Tsutsumi Y. Extraprostatic localization of prostatic acid phosphatase and prostate-specific antigen: distribution in cloacogenic glandular epithelium and sex-dependent expression in human anal gland. *Hum Pathol* 1990;21:1108-11. MEDLINE Abstract

30. Iwakiri J, Grandbois K, Wehner N, Graves HCB, Stamey T. An analysis of urinary prostate specific antigen before and after radical prostatectomy: evidence for secretion of prostate specific antigen by the periurethral glands. *J Urol* 1993;149:783-6. MEDLINE Abstract

31. Van Krieken TH. Prostate marker immunoreactivity in salivary gland neoplasms. A rare pitfall in immunohistochemistry. *Am J Surg Pathol* 1990;17:410-4.

32. Wernett N, Albrech M, Sesterhum I, Goebbels R, Bonkhoff H, Seiz G, et al. The 'female prostate': location, morphology, immunohistochemical characteristics and significance. *Eur Urol* 1992;17:64-9.

33. Zarghami N, D'Costa M, Tsuyuki D, Asa SL, Diamandis EP. Expression of the prostate specific antigen gene by lung tissue. *Clin Cancer Res* 1997;3:1201-6.

34. Clements J, Mukhtar A. Glamdular kallikreins and prostate-specific antigen are expressed in human endometrium. *J Clin Endocrinol Metab* 1994;78:1536-9. MEDLINE Abstract

35. Levesque M, Yu H, D'Costa M, Tadross L, Diamandis EP. Immunoreactive prostate specific antigen in lung tumors. *J Clin Lab Anal* 1995;9:375-9. MEDLINE Abstract

---

Received July 13, 1998; accepted August 31, 1998
For reprints and all correspondence: Kazuhiko Uchida, Department of Biochemistry and Molecular Oncology, Institute of Basic Medical Sciences, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba, Ibaraki 305-8575, Japan. E-mail: kzuchida{at}md.tsukuba.ac.jp
Abbreviations: AFP, [alpha]-fetoprotein; PSA, prostate-specific antigen; RT-PCR, reverse transcriptase polymerase chain reaction; HCC, hepatocellular carcinoma; AGPC, acid guanidinium thiocyanate-phenol-chloroform; MuLV, murine leukemia virus

---

A7

JOURNAL OF VIROLOGY, Dec. 1998, p. 10044–10049 Vol. 72, No. 12
0022-538X/98/$04.00+0

Detection and Sequence Analysis of Borna Disease Virus p24 RNA from Peripheral Blood Mononuclear Cells of Patients with Mood Disorders or Schizophrenia and of Blood Donors

YASUHIDE IWATA,[1]† KAZUO TAKAHASHI,[1]* XIE PENG,[2]‡ KOJI FUKUDA,[2] KOEI OHNO,[1] TSUGUHIRO OGAWA,[1] KENJI GONDA,[1] NORIO MORI,[2] SHIN-ICHI NIWA,[3] AND SHIRO SHIGETA[1]

Department of Microbiology[1] and Department of Neuropsychiatry,[3] School of Medicine, Fukushima Medical University, Fukushima-shi, Fukushima, 960-1295, and Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu-shi, Shizuoka, 431-3192,[2] Japan

Received 27 April 1998/Accepted 24 August 1998

Borna disease virus (BDV) p24 RNA was detected in the peripheral blood mononuclear cells (PBMCs) of psychiatric patients and blood donors by nested reverse transcriptase PCR (RT-PCR). The prevalences of BDV p24 RNA in patients with mood disorders (4%) and schizophrenia (4%) were not significantly different from that in blood donors (2%). This finding was inconsistent with previous reports that showed either a high prevalence or absence of BDV p24 RNA in patients with psychiatric disorders. The differences in BDV p24 RNA prevalence in these studies may be due to differences in the criteria for positivity, the number of PBMCs used for RNA extraction, or the amount of RNA tested for nested RT-PCR or to laboratory contamination. Sequence analysis of BDV p24 RNA from the PBMCs of patients and blood donors showed a high nucleotide sequence conservation but definite nucleotide mutations compared with horse BDV p24 RNA sequences. In comparison with human BDV p24 RNA sequences previously reported from Japan and Germany, there were several positions with silent nucleotide mutations among these clones.

Epidemiological data suggests that not only genetic factors but also environmental factors may play an important role in the etiology of psychiatric disorders, including schizophrenia and mood disorders (10, 40). The association of viral infections, such as cytomegalovirus, herpes simplex virus, and influenza virus, with psychiatric disorders has been implied (1, 18, 29); however, the role of viral etiology in psychiatric disorders remains unclear.

Borna disease (BD) is an acute meningoencephalitis of horses and sheep caused by BD virus (BDV) (4). BDV is a noncytolytic neurotrophic virus that infects several vertebrate species, including birds and primates (9, 27, 37, 38, 43), and causes central nervous system dysfunction with various manifestations depending on the age, immune status, and species of the host (26, 28, 39). For example, BDV induces a severe cellular immune response to infected cells in the brains of adult rats with movement and behavioral abnormalities, or a persistent infection with only cognitive impairment in neonatal rats (3). The viral nucleic acids and antigens are found in limbic pyramidal and extrapyramidal motor systems (8, 25, 36). BDV is a nonsegmented, negative, single-stranded RNA virus (7, 13, 41). The 8.9-kb RNA genome of BDV contains five major open reading frames (ORFs), three of which encode viral polypeptides of 40, 24, and 14.5 kDa (11, 30). RNA splicing is used for the expression of BDV-specific mRNA (12).

The wide host range of the virus and behavioral disturbances in animals with BD suggest that BDV infection may be associated with human psychiatric disorders (2, 32). Seroepidemiological data and the detection of BDV RNA in the peripheral blood mononuclear cells (PBMCs) of psychiatric patients have also suggested a possible involvement of BDV in human psychiatric disorders (5, 20, 22, 34, 41, 42). However, there is controversy over the prevalence of BDV antibody and BDV RNA in the PBMCs of patients with psychiatric disorders. Furthermore, recently the isolation of infectious BDV from the PBMCs of psychiatric patients and the sequencing of the major part of the viral genome have been reported (6). There is also controversy as to whether the human BDV p24 sequence is highly conserved or variable with respect to the horse BDV sequence.

In the present study, to confirm the prevalence of BDV infection in psychiatric patients, we have investigated the prevalence of BDV p24 RNA in the PBMCs of patients with mood disorders or schizophrenia and in those of blood donors by nested reverse transcriptase PCR (RT-PCR). Furthermore, sequence analysis of the human BDV p24 cDNA showed high nucleotide sequence conservation of p24 but a distinct nucleotide mutation in comparison with horse BDV sequences.

MATERIALS AND METHODS

Subjects. The number and profiles of patients and blood donors included in this study are summarized in Table 1. The patients consisted of 49 with mood disorders and 77 with schizophrenia. All patients met DSM-IV criteria (American Psychiatric Association, 1994) for the diagnosis of mood disorders and schizophrenia. The blood donors were matched by age and sex to the patients. This study was approved by the Ethical Committee of Fukushima Medical University, and all patients gave their informed consent.

Preparation of RNA. For each subject, PBMCs were isolated from 15 ml of blood by using heparin or EDTA and centrifugation on Ficoll-Conray solution (density, 1.077 g/ml). Total cellular RNA was prepared from 10^7 PBMCs with an

* Corresponding author. Mailing address: Department of Microbiology, School of Medicine, Fukushima Medical University, 1 Hikarigaoka, Fukushima-shi, Fukushima, 960-1295, Japan. Phone: 81-24-548-2111, ext. 2162. Fax: 81-24-548-5072. E-mail: k-tak@cc.fmu.ac.jp.

† Present address: Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu-shi, Shizuoka, 431-3192, Japan.

‡ Present address: Department of Neurology and Psychiatry, First Affiliated Hospital, Chongqing University of Medical Sciences, Chongqing 400016, People's Republic of China.

TABLE 1. Detection of BDV p24 RNA in PBMCs of psychiatric patients and blood donors

Source	Age (mean yr ± SD)	No. positive/no. tested (% positive)				
		Sex		Treatment		Total
		Male	Female	In-patient	Out patient	
Psychiatric patients						
Mood disorder	47.5 ± 12.6	1/25 (4)	1/24 (4)	1/30 (5)	1/19 (7)	2/49 (4)
Schizophrenia	45.4 ± 10.3	0/45 (0)	3/32 (9)	2/69 (5)	1/8 (8)	3/77 (4)
Blood donors	45.2 ± 10.2	2/49 (2)	0/35 (0)			2/84 (2)

RNA extraction kit (Isogen; Nippon-Gene, Tokyo, Japan) according to the manufacturer's manual. The approximate concentration of extracted RNA was determined by spectrophotometry. Total RNA was also isolated from Madin-Darby canine kidney (MDCK) cells persistently infected with BDV (BDV-MDCK) originally isolated from a horse and was used as a positive control for nested RT-PCR. The BDV-MDCK was kindly provided by R. Rott (19).

Detection of BDV p24 RNA by nested RT-PCR. BDV p24 RNA was detected by nested RT-PCR. Approximately 1 μg of cellular RNA was reverse transcribed, and the first PCR was performed with a thermostable r*Tth* RT RNA PCR kit (Perkin-Elmer Co., Foster City, Calif.) with BDV p24-specific primers as described by Kishi et al. (20). Primer pairs used for the reverse transcription and first PCR were 5′-TGACCCAACCAGTAGACCA-3′ (P24-OF; nucleotides [nt] 1387 to 1405) and 5′-GTCCCATTCATCCGTTGTC-3′ (P24-OR; nt 1865 to 1847). The second pair of primers were 5′-TCAGACCCAGACCAGCGAA-3′ (P24-MF; nt 1443 to 1461) and 5′-AGCTGGGGATAAATGCGCG-3′ (P24-MR; nt 1834 to 1816). The numbering of the BDV nucleotide sequence followed that reported for the horse BDV sequence adapted in C6 cells (C6BV) (11). Reverse transcription was performed at 70°C for 15 min followed by 60°C for 15 min in a 10-μl (total volume) mixture; after reverse transcription, the first PCR was performed in a 50-μl mixture according to the manufacturer's instructions. The conditions of the first and second PCRs were as follows: 92°C for 1 min (1 cycle) and 40 cycles of 92°C for 1 min, 55°C for 1 min, and 75°C for 1 min in a thermal cycler (OmniGene; Hybaid, Teddington, United Kingdom). For the second PCR, 0.5 μl of the first PCR product was amplified in a 25-μl mixture containing 0.5 μM (each) nested primers (P24-MF and P24-MR), 0.2 mM (each) deoxynucleoside triphosphates, 10 mM Tris-HCl (pH 8.9), 1.5 mM $MgCl_2$, 80 mM KCl, 500 μg of bovine serum albumin/ml, 0.1% sodium cholate, 0.1% Triton X-100, and 0.5 U of *Tth* polymerase (Toyobo, Osaka, Japan). The PCR products were separated by 1.4% agarose gel electrophoresis and stained with ethidium bromide. The specificity of the amplification products was demonstrated by Southern blot hybridization with an alkaline phosphatase (ALP)-labeled oligonucleotide specific for BDV p24 nucleotides (5′-ALP-TCAGCGGTGCGACCA CTCCGATAGC-3′) (ALP-P24; nt 1637 to 1661).

To evaluate the quality of the RNA extraction, RT-PCR for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was carried out with 10 ng of total RNA as described previously (16). To prevent contamination of the amplified DNA, PCR was performed according to a strict protocol recommended by Kwok and Higuchi (23). RNA samples and the PCR mixture were prepared in a biosafety hood in an exclusive room where the amplified DNA had never been used. Amplification reactions and analysis of PCR products were performed in a room separate from that used for RNA preparation. Filter tips were used to avoid aerosols that might have contained amplified sample DNA. To confirm the presence of BDV RNA, nested RT-PCR was repeated in the positive cases. We regarded a sample as positive if it showed positive twice. In some positive cases, nested RT-PCR without the RT enzyme in the reverse transcription step were performed to rule out contamination with amplicons of BDV p24 sequences.

Generation of BDV p24 RNA fragments. The first PCR fragment of BDV p24 (479 bp) amplified from total RNA of BDV-infected MDCK cells was purified and ligated into pGEM-T Easy vector (Promega, Madison, Wis.). The cloned vector was linearized with *Sal*I and in vitro transcribed with T7 RNA polymerase (Life Technologies, Inc., Rockville, Md.) to generate BDV p24 RNA fragments, followed by DNase I (Life Technologies, Inc., Rockville, Md.) digestion of DNA template. The RNA was extracted with phenol and precipitated with ethanol. The concentration of generated RNA was estimated by spectrophotometry and by ethidium bromide staining in comparison with a known amount of tRNA.

Statistical analysis. Differences in the prevalence of BDV p24 RNA between subject subgroups were analyzed by using Fisher's exact probability method.

Cloning and sequencing of PCR products. PCR products were purified and cloned in pGEM-T vectors. Three to five clones from each PCR product were sequenced on both strands by the dideoxy chain termination method with T7 or p24-MF and SP6 primers and a SequiTherm Long-Read Cycle-Sequencing Kit-LC (Epicentre Technologies, Madison, Wis.) and a DNA-sequencing system (model 4000 automated DNA sequencer; LI-COR, Lincoln, Nebr.). Sequence analyses were performed with MacMolly Tetra (Soft Gene GmbH, Berlin, Germany).

Mutations experimentally induced by nested RT-PCR. To evaluate the sequence heterogeneity of BDV in humans, it is necessary to distinguish between real nucleotide substitutions and those experimentally induced by the nested RT-PCR assay. To determine the experimentally induced mutation frequency, two different nested RT-PCR tests were performed with 100 copies of in vitro-transcribed BDV p24 RNA, which is the limit of sensitivity of our nested RT-PCR. Both second-PCR products were cloned in pGEM-T vectors, and each group of four clones was sequenced. Mutation frequencies among four clones (intragroup) and between two groups (intergroup) were determined.

RESULTS

Sensitivity of nested RT-PCR for BDV p24 RNA. To determine the sensitivity of nested RT-PCR for BDV p24, the in vitro-transcribed BDV p24 RNA fragment was 10-fold serially diluted with tRNA solution (1 μg/μl) and tested by nested RT-PCR. BDV p24 RNA could be consistently detected from 100 molecules of the generated BDV p24 RNA fragment by nested RT-PCR and Southern blot hybridization (Fig. 1). Similarly, 100 fg of total RNA of BDV-infected MDCK cells was constantly detected. The nested RT-PCR specifically yielded a predicted 392-bp DNA fragment. In each RT-PCR experiment, a positive control containing 100 fg of RNA from BDV-MDCK-infected cells was included to evaluate the efficacy of the nested RT-PCR, and it was always found positive.

Detection of BDV p24 RNA sequences in PBMCs from psychiatric patients and blood donors. To evaluate the prevalence of BDV RNA in psychiatric patients, RNA samples from the PBMCs were tested by nested RT-PCR. As shown in Table 1, BDV p24-specific DNA fragments were detected by Southern blot analysis in 2 of 49 (4%) patients with mood disorders, 3 of 77 (4%) patients with schizophrenia, and 2 of 84 (2%) blood donors. All positive samples were confirmed to be positive by repeating the nested RT-PCR. Figure 2 illustrates representative results obtained with samples from psychiatric patients. A 598-bp GAPDH fragment was amplified as an internal control in all samples analyzed, suggesting that the quality of the RNA tested was sufficient. There were no significant differences in the prevalence of BDV RNA among the three groups. To rule out possible contamination with amplified BDV p24 sequences in the positive cases, nested RT-PCR without RT enzyme in a reverse transcription step was performed with the two samples of the control group, and the reaction was found to be RT dependent (data not shown). However, in the positive cases from the patient groups, a nested RT-PCR assay without the reverse transcription step was not performed because of a lack of RNA samples from the patients.

Sequence analysis of the BDV p24 PCR fragments derived from human PBMCs. To determine the sequences of the human BDV p24 fragments representing 55% of the p24 gene, and to compare them with reported horse and human BDV sequences, PCR products from two patients with mood disorders, three patients with schizophrenia, and two blood donors were cloned and three to five clones of each PCR product were

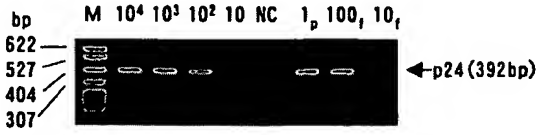


FIG. 1. Sensitivity of nested RT-PCR for BDV p24 RNA. Serially diluted in vitro-transcribed BDV p24 RNA fragments (10^4, 10^3, 10^2, and 10 copies) and RNA from BDV-MDCK cells (1 pg [1_p], 100 fg [100_f], and 10 fg (10_f]) were amplified by nested RT-PCR and analyzed by 1.4% agarose gel electrophoresis and ethidium bromide staining. Deionized distilled water was used as a negative control (NC). M, molecular size markers.

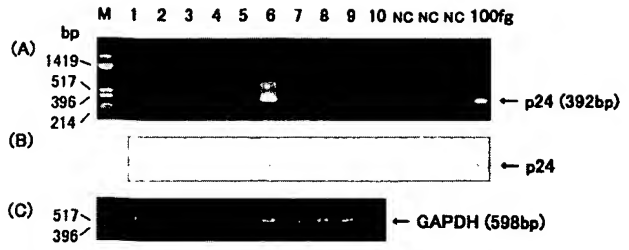


FIG. 2. Detection of BDV p24 RNA in PBMCs of psychiatric patients by nested RT-PCR. Representative results for 10 samples from patients are shown in lanes 1 to 10. PCR products of BDV p24 were analyzed by 1.4% agarose gel electrophoresis and ethidium bromide staining (A) and by Southern blot hybridization with a BDV p24-specific oligonucleotide labeled with ALP (B). One hundred femtograms of RNA extracted from BDV-infected MDCK cells was used as a positive control (100fg), and three independent tubes of distilled water were used as negative controls (NC [three lanes]). As a control for RNA quality, cellular GAPDH mRNA was analyzed by RT-PCR and ethidium bromide staining (C). BDV p24- and GAPDH-specific fragments are indicated at 392 and 598 bp, respectively. M, molecular size markers.

sequenced. The results of the sequence analysis are presented in Fig. 3 along with the sequences of horse BDV strain V (35), C6BV (11), and BDV-MDCK. We sequenced 15 clones from BDV-MDCK, and a predominant consensus sequence is shown in Fig. 3. In total, 30 different p24 clones derived from seven different PBMC samples were analyzed. Cloning and sequencing of the p24 fragments, corresponding to nt 1480 to 1814 of ORF p24, confirmed their BDV specificity and showed a high level of sequence conservation with the sequences of three horse BDV strains. The sequence analysis of human BDV and BDV-MDCK showed the lowest divergency (0 to 1.79%), and the divergency between human BDV and C6BV (3.58 to 5.07%) was the highest. The divergency with strain V was intermediate (1.79 to 3.28%). As shown in Fig. 3, no position with a human-specific mutation was found in all clones from all individuals. However, compared to BDV-MDCK sequences, we identified two positions with mutations, at nt 1535 and 1617. The first silent mutation (C→T), at nt 1535 with no amino acid change, was found in all individuals except one patient with a mood disorder (D1) and blood donor H1-5. The second mutation (G→A), at nt 1617, found in one patient with mood disorders (D1), had a conservative amino acid change (Val→Met). One-point substitutions with no clusters were randomly distributed in human BDV p24 sequences. Interindividual divergency was slightly higher than intraindividual divergency (0 to 2.69 versus 0 to 2.09%).

Experimentally induced nucleotide mutations during nested RT-PCR for in vitro-transcribed BDV p24 RNA. To determine the experimentally induced mutation frequency during nested RT-PCR, two different nested RT-PCR tests were performed with 100 copies of in vitro-transcribed BDV p24 RNA, and four clones of each second-PCR product were sequenced. Both the intragroup divergencies among four clones and the intergroup divergencies between two nested RT-PCR tests were 0 to 1.49%.

DISCUSSION

In the present study, we have detected BDV RNA in the PBMCs of psychiatric patients and blood donors by nested RT-PCR. The prevalences of BDV p24 RNA in patients with mood disorders (4%) and schizophrenia (4%) were not significantly different from that in blood donors (2%). We performed nested RT-PCR experiments carefully to avoid BDV DNA contamination, as described in Materials and Methods. Three water samples used as negative controls were always negative in every PCR assay. Furthermore, the nucleotide (T) at nt 1535 of human BDV p24 RNA from patient PBMCs was clearly different from that (C) at the same position in a horse BDV-MDCK sequence which was used as the positive control in nested RT-PCR assays. In addition, RT dependency was confirmed in the two positive cases among blood donors, although in the five positive cases in the patient groups RT dependency could not be tested because of a lack of RNA samples. These findings suggest that we can rule out contamination with horse BDV p24 RNA or amplified BDV DNA sequences in our nested RT-PCR system.

In previous studies, there has been controversy as to the prevalence of BDV RNA in the PBMCs of psychiatric patients. Kishi et al. reported a significantly high prevalence (37%) of BDV RNA in Japanese psychiatric patients compared with that in healthy individuals (6.5%) (20). However, a method of nested RT-PCR almost the same as that they established was used in our study (except for the RT-PCR kit), and the sensitivity of our nested RT-PCR was slightly higher. The reason for the difference in prevalence may be due to differences in the criteria for a positive result. Sauder et al. also reported a high prevalence (38.5%) of BDV RNA in psychiatric patients in Homburg, Germany (34). The sensitivity of the nested RT-PCR, the number of PBMCs used for RNA extraction, and the amount of RNA used for RT-PCR were similar to those in our method. One of the reasons for the difference in prevalence may be geographical. On the other hand, a low prevalence (1.9%) of BDV p24 RNA in psychiatric patients and no viral RNA in control subjects have been reported in Japan (22). The difference between the prevalences in our study and theirs may be due to the lower sensitivity of their single RT-PCR assay. Richt et al. (31) and Lieb et al. (24) reported that there was no evidence for the presence of BDV RNA in PBMCs from either psychiatric patients or normal controls. Since the sensitivity of the nested RT-PCR performed by Lieb et al. was similar to ours (100 molecules), the difference in prevalence may be due to the difference in the number of PBMCs used for RNA extraction and/or the amount of RNA tested for nested RT-PCR. The difference in prevalence in the study of Richt et al. may be due to differences in the number of subjects and/or the sensitivity of nested RT-PCR. Other reasons which may account for the differences in prevalence in previous studies are different criteria for positivity and laboratory contamination of amplified BDV DNA. For a precise comparison of the prevalences of BDV RNA, standardization of the PCR method (e.g., the number of PBMCs used for RNA extraction, the sensitivity of the PCR, and the criteria for positivity) is necessary.

Sequence analysis of BDV p24 RNA from the PBMCs of patients and blood donors showed a high nucleotide sequence conservation with horse BDV p24 sequences (Fig. 3). This high homology is consistent with two previous reports of psychiatric patients from Germany (15, 34) and inconsistent with one previous report from Japan (21). The human BDV sequences in our study were homologous with those of horse BDV in the following decreasing order: BDV-MDCK, strain V, and C6BV. In previous reports on the divergency between human and horse BDV p24 sequences, the BDV p24 sequences of psychiatric patients in Berlin, Germany, exhibited higher conservation with strain V than with C6BV, while the sequences of patients in Homburg, Germany, showed higher homology with C6BV than with strain V (15, 34). The BDV sequence from Kagoshima, Japan (22), was identical to that of the BDV-MDCK sequence and more homologous with strain V. These findings may suggest the existence of heterogeneity in human BDV sequences.

Figure 4 summarizes nucleotide and amino acid mutations among previously reported human BDV p24 sequences (335-

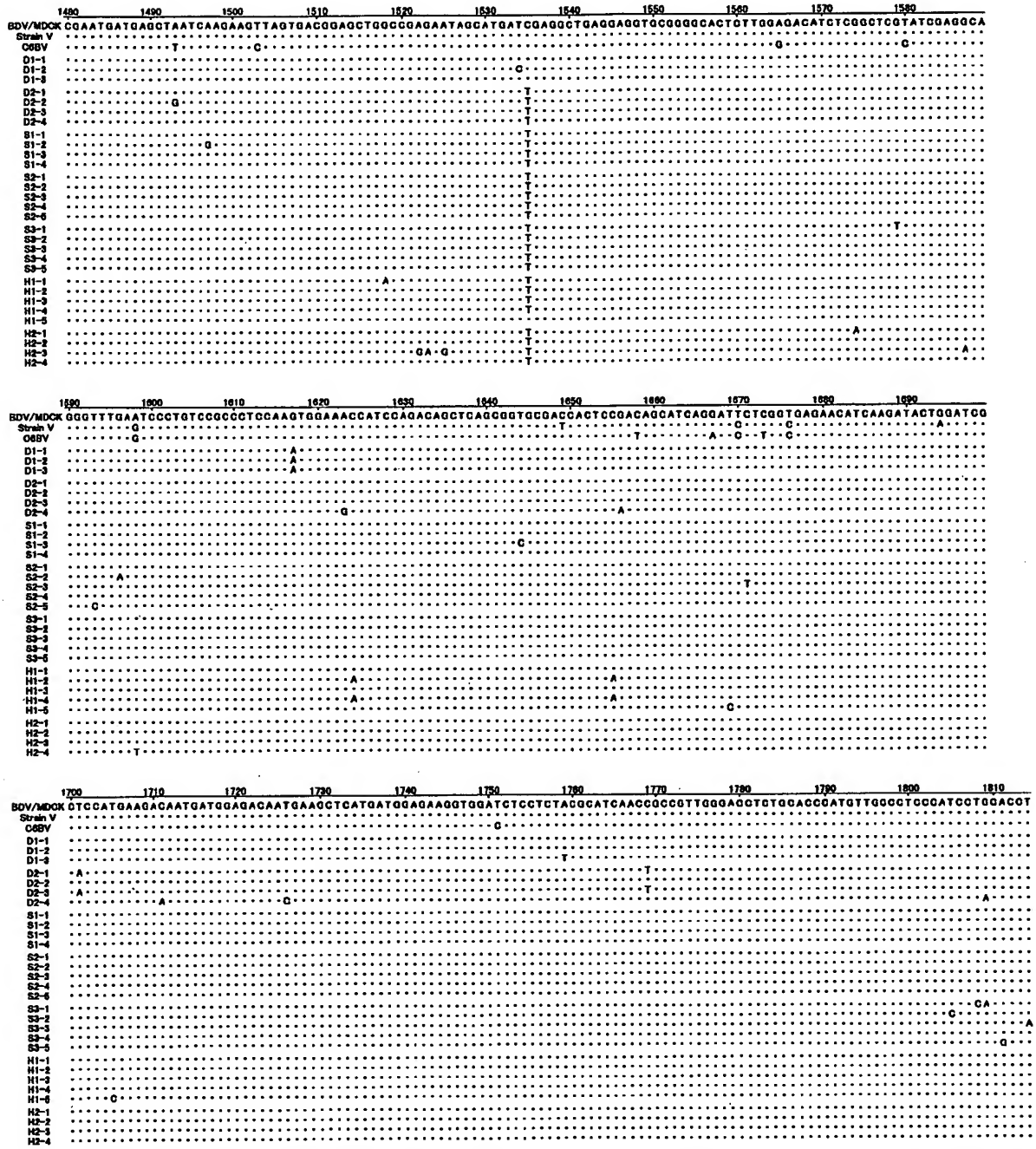


FIG. 3. Nucleotide sequences of BDV p24 cDNA clones isolated from PBMCs of two patients with mood disorders (D1 and D2), three patients with schizophrenia (S1, S2, and S3), and two blood donors (H1 and H2) in comparison with those of horse BDVs (BDV-MDCK, strain V, and C6BV). The nucleotide sequence of BDV-MDCK was determined in this study, and the sequences of strain V (35) and C6BV (11) were based on those published previously. The nucleotide numbers above the sequence follow the numbering previously reported for C6BV (11). The dots indicate nucleotide identity with the BDV-MDCK consensus sequence.

bp sequence) in comparison with horse BDV sequences. Nucleotide sequences which are not presented in Fig. 4 are identical among human BDV sequences. Amino acid substitutions which are specific and common to all human BDV sequences were not observed. However, at the nucleotide level, we detected three silent substitutions, at positions 1598, 1670, and 1676, which can distinguish between Japanese and German clones. Furthermore, there are eight positions with silent mutations without amino acid changes (nt 1493, 1503, 1565, 1580, 1658, 1667, 1673, and 1751) which are Homburg specific and identical to those of C6BV. C-to-T silent substitution at nt 1535 without an amino acid change was found in all of the Fukushima clones except those from patient D1. C-to-T and G-to-A silent substitutions at nt 1649 and 1694 were found only in Berlin clones, which were identical to the horse strain V sequence. These findings suggest that human BDV p24 sequences are conserved in general but some sequences differ among clones in different areas.

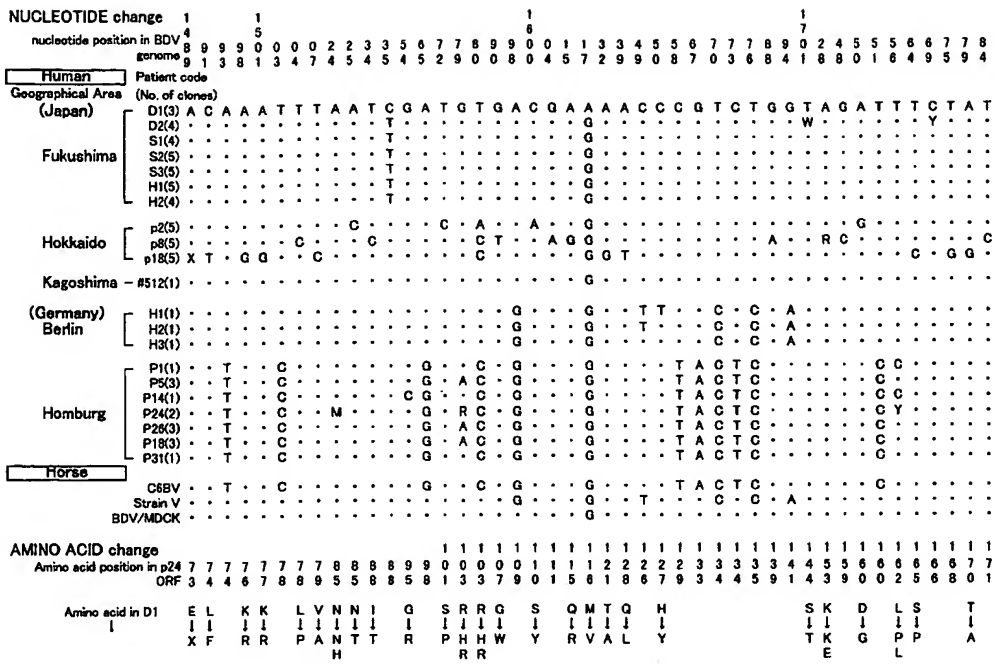


FIG. 4. Nucleotide mutations in the partial human BDV p24 sequences derived from psychiatric patients in different geographical areas. The nucleotide numbers above the sequence follow the numbering of the previously reported C6BV sequence (11). The dots indicate nucleotide identity with the sequence of D1. The numbers in parentheses indicate the number of clones sequenced. M, R, W, Y, and X indicate A or C, A or G, A or T, C or T, and deletion, respectively. The sequences obtained in Hokkaido, Kagoshima, Berlin, and Homburg are from references 21, 22, 15, and 34, respectively. Amino acid changes corresponding to nucleotide mutations are shown at the bottom of the figure.

Interindividual and intraindividual divergencies among the patients in our study were 0 to 2.69% and 0 to 2.09%, respectively. To clarify whether these mutations were experimentally induced or were due to sequence heterogeneity already present in the individuals, we also examined the mutation frequency during nested RT-PCR by using in vitro-transcribed BDV p24 RNA. Both intergroup and intragroup divergencies in nested RT-PCR were 0 to 1.49% in 335 bp. Considering these mutation frequencies, most of the one-point substitutions, except those at nt 1535 and 1617, which were randomly distributed in the human BDV sequences, are likely due to experimentally induced mutations. The sequences from psychiatric patients in Hokkaido, Japan, have been reported to be significantly variable (21). It was pointed out that the nested RT-PCR procedure used by the Japanese researchers produced several mutations, probably due to the negative effect of manganese during DNA polymerization (34). In our RT-PCR system, manganese was chelated before the first PCR to reduce the number of artificial mutations.

Although the results of the present study of the detection of BDV RNA in PBMCs did not confirm the association of BDV infection with mood disorders and schizophrenia, the existence of BDV or a BDV-related agent was shown in some humans. In addition, the possible existence of heterogeneity in human BDV sequences was suggested. Recently it was reported that BDV RNA was detected in the brains of both psychiatric patients (14, 33) and healthy individuals (17). To clarify the association of BDV with psychiatric disorders, it is important not only to detect the BDV genome in the brains of psychiatric patients but also to elucidate the effect of BDV infection on brain function. Further studies are necessary to clarify the role of BDV infection in the etiology and pathogenesis of psychiatric disorders.

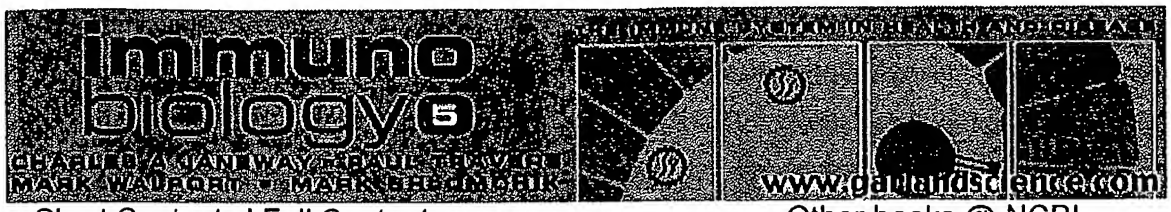
ACKNOWLEDGMENTS

We thank Yuichi Endo for valuable discussions; Francis A. Ennis for reviewing the manuscript; Hiroki Suzuki, Kiyoshi Ariga, Koichi Osonoe, Minako Sato, Mitsuhiro Ito, Noboru Yokoyama, Shin-ichi Ogata, and Yoshihiko Numata for obtaining samples from patients; and Chiharu Takahashi, Manabu Kikuchi, Yumiko Kanno, Sachiko Sakai, Kazuhiro Mochizuki, and Yasuki Takeuchi for technical assistance.

REFERENCES

1. **Albrecht, P., E. F. Torrey, E. Boone, J. T. Hicks, and N. Daniel.** 1980. Raised cytomegalovirus-antibody level in cerebrospinal fluid of schizophrenic patients. Lancet ii:769–772.
2. **Amsterdam, J. D., A. Winokur, W. Dyson, S. Herzog, F. Gonzalez, R. Rott, and H. Koprowski.** 1985. Borna disease virus. A possible etiologic factor in human affective disorders? Arch. Gen. Psychiatry 42:1093–1096.
3. **Bautista, J. R., S. A. Rubin, T. H. Moran, G. J. Schwartz, and K. M. Carbone.** 1995. Developmental injury to the cerebellum following perinatal Borna disease virus infection. Dev. Brain Res. 90:45–53.
4. **Binz, T., J. Lebelt, H. Niemann, and K. Hagenau.** 1994. Sequence analyses of the p24 gene of Borna disease virus in naturally infected horse, donkey and sheep. Virus Res. 34:281–289.
5. **Bode, L., R. Ferszt, and G. Czech.** 1993. Borna disease virus infection and affective disorders in man. Arch. Virol. 7(Suppl.):159–167.
6. **Bode, L., R. Durrwald, F. A. Rantam, R. Ferszt, and H. Ludwig.** 1996. First isolates of infectious human Borna disease virus from patients with mood disorders. Mol. Psychiatry 1:200–212.
7. **Briese, T., A. Schneemann, A. J. Lewis, Y. S. Park, S. Kim, H. Ludwig, and W. I. Lipkin.** 1994. Genomic organization of Borna disease virus. Proc. Natl. Acad. Sci. USA 91:4362–4366.
8. **Carbone, K. M., C. S. Duchala, J. W. Griffin, A. L. Kincaid, and O. Narayan.** 1987. Pathogenesis of Borna disease in rats: evidence that intra-axonal spread is the major route for virus dissemination and the determinant for disease incubation. J. Virol. 61:3431–3440.
9. **Carbone, K. M., S. A. Rubin, A. M. Sierra-Honigmann, and H. M. Lederman.** 1993. Characterization of a glial cell line persistently infected with Borna disease virus (BDV): influence of neurotrophic factors on BDV protein and RNA expression. J. Virol. 67:1453–1460.
10. **Crow, T. J.** 1983. Is schizophrenia an infectious disease? Lancet i:173–175.

11. **Cubitt, B., C. Oldstone, and J. C. de la Torre.** 1994. Sequence and genome organization of Borna disease virus. J. Virol. 68:1382–1396.
12. **Cubitt, B., C. Oldstone, J. Valcarcel, and J. C. de la Torre.** 1994. RNA splicing contributes to the generation of mature mRNAs of Borna disease virus, a non-segmented negative strand RNA virus. Virus Res. 34:69–79.
13. **de la Torre, J. C., K. M. Carbone, and W. I. Lipkin.** 1990. Molecular characterization of the Borna disease agent. Virology 179:853–856.
14. **de la Torre, J. C., D. Gonzalez-Dunia, B. Cubitt, M. Mallory, N. Mueller-Lantzsch, F. A. Grasser, L. A. Hansen, and E. Masliah.** 1996. Detection of borna disease virus antigen and RNA in human autopsy brain samples from neuropsychiatric patients. Virology 223:272–282.
15. **de la Torre, J. C., L. Bode, R. Durrwald, B. Cubitt, and H. Ludwig.** 1996. Sequence characterization of human Borna disease virus. Virus Res. 44:33–44.
16. **Gao, X., K. V. Honn, D. Grignon, W. Sakr, and Y. Q. Chen.** 1993. Frequent loss of expression and loss of heterozygosity of the putative tumor suppressor gene DCC in prostatic carcinomas. Cancer Res. 53:2723–2727.
17. **Haga, S., M. Yoshimura, Y. Motoi, K. Arima, T. Aizawa, K. Ikuta, M. Tashiro, and K. Ikeda.** 1997. Detection of Borna disease virus genome in normal human brain tissue. Brain Res. 770:307–309.
18. **Halonen, P. E., R. Rimon, K. Arohonka, and V. Jantti.** 1974. Antibody levels to herpes simplex type I, measles and rubella viruses in psychiatric patients. Br. J. Psychiatry 125:461–465.
19. **Herzog, S., and R. Rott.** 1980. Replication of Borna disease virus in cell cultures. Med. Microbiol. Immunol. 168:153–158.
20. **Kishi, M., T. Nakaya, Y. Nakamura, Q. Zhong, K. Ikeda, M. Senjo, M. Kakinuma, S. Kato, and K. Ikuta.** 1995. Demonstration of human Borna disease virus RNA in human peripheral blood mononuclear cells. FEBS Lett. 364:293–297.
21. **Kishi, M., Y. Arimura, K. Ikuta, Y. Shoya, P. K. Lai, and M. Kakinuma.** 1996. Sequence variability of Borna disease virus open reading frame II found in human peripheral blood mononuclear cells. J. Virol. 70:635–640.
22. **Kubo, K., T. Fujiyoshi, M. M. Yokoyama, K. Kamei, J. A. Richt, B. Kitze, S. Herzog, M. Takigawa, and S. Sonoda.** 1997. Lack of association of Borna disease virus and human T-cell leukemia virus type 1 infections with psychiatric disorders among Japanese patients. Clin. Diagn. Lab. Immunol. 4:189–194.
23. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature 339:237–238.
24. **Lieb, K., W. Hallensleben, M. Czygan, L. Stitz, P. Staeheli, and the Bornavirus Study Group.** 1997. No Borna disease virus-specific RNA detected in blood from psychiatric patients in different regions of Germany. Lancet 350: 1002.
25. **Lipkin, W. I., G. H. Travis, K. M. Carbone, and M. C. Wilson.** 1990. Isolation and characterization of Borna disease agent cDNA clones. Proc. Natl. Acad. Sci. USA 87:4184–4188.
26. **Lipkin, W. I., A. Schneemann, and M. V. Solbrig.** 1995. Borna disease virus: implications for human neuropsychiatric illness. Trends Microbiol. 3:64–69.
27. **Lundgren, A. L., W. Zimmermann, L. Bode, G. Czech, G. Gosztonyi, R. Lindberg, and H. Ludwig.** 1995. Staggering disease in cats: isolation and characterization of the feline Borna disease virus. J. Gen. Virol. 76:2215–2222.
28. **Narayan, O., S. Herzog, K. Frese, H. Scheefers, and R. Rott.** 1983. Behavioral disease in rats caused by immunopathological responses to persistent borna virus in the brain. Science 220:1401–1403.
29. **O'Callaghan, E., P. Sham, N. Takei, G. Glover, and R. M. Murray.** 1991. Schizophrenia after prenatal exposure to 1957 A2 influenza epidemic. Lancet 337:1248–1250.
30. **Pyper, J. M., J. A. Richt, L. Brown, R. Rott, O. Narayan, and J. E. Clements.** 1993. Genomic organization of the structural proteins of borna disease virus revealed by a cDNA clone encoding the 38-kDa protein. Virology 195:229–238.
31. **Richt, J. A., R. C. Alexander, S. Herzog, D. C. Hooper, R. Kean, S. Spitsin, K. Bechter, R. Schuttler, H. Feldmann, A. Heiske, Z. F. Fu, B. Dietzschold, R. Rott, and H. Koprowski.** 1997. Failure to detect Borna disease virus infection in peripheral blood leukocytes from humans with psychiatric disorders. J. Neurovirol. 3:174–178.
32. **Rott, R., S. Herzog, B. Fleischer, A. Winokur, J. Amsterdam, W. Dyson, and H. Koprowski.** 1985. Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. Science 228:755–756.
33. **Salvatore, M., S. Morzunov, M. Schwemmle, W. I. Lipkin, and the Bornavirus Study Group.** 1997. Borna disease virus in brains of North American and European people with schizophrenia and bipolar disorder. Lancet 349: 1813–1814.
34. **Sauder, C., A. Müller, B. Cubitt, J. Mayer, J. Steinmetz, W. Trabert, B. Ziegler, K. Wanke, N. Mueller-Lantzsch, J. C. de la Torre, and F. A. Grässer.** 1996. Detection of Borna disease virus (BDV) antibodies and BDV RNA in psychiatric patients: evidence for high sequence conservation of human blood-derived BDV RNA. J. Virol. 70:7713–7724.
35. **Schneider, P. A., T. Briese, W. Zimmermann, H. Ludwig, and W. I. Lipkin.** 1994. Sequence conservation in field and experimental isolates of Borna disease virus. J. Virol. 68:63–68.
36. **Solbrig, M. V., G. F. Koob, J. H. Fallon, and W. I. Lipkin.** 1994. Tardive dyskinetic syndrome in rats infected with Borna disease virus. Neurobiol. Dis. 1:111–119.
37. **Sprankel, H., K. Richarz, H. Ludwig, and R. Rott.** 1978. Behavior alterations in tree shrews (Tupaia glis, Diard 1820) induced by Borna disease virus. Med. Microbiol. Immunol. 165:1–18.
38. **Stitz, L., H. Krey, and H. Ludwig.** 1981. Borna disease in rhesus monkeys as a model for uveo-cerebral symptoms. J. Med. Virol. 6:333–340.
39. **Stitz, L., T. Bilzer, J. A. Richt, and R. Rott.** 1993. Pathogenesis of Borna disease. Arch. Virol. 7(Suppl.):135–151.
40. **Torrey, E. F., J. Miller, R. Rawlings, and R. H. Yolken.** 1997. Seasonality of births in schizophrenia and bipolar disorder: a review of the literature. Schizophr. Res. 28:1–38.
41. **VandeWoude, S., J. A. Richt, M. C. Zink, R. Rott, O. Narayan, and J. E. Clements.** 1990. A borna virus cDNA encoding a protein recognized by antibodies in humans with behavioral diseases. Science 250:1278–1281.
42. **Waltrip, R. W., II, R. W. Buchanan, W. T. Carpenter, Jr., B. Kirkpatrick, A. Summerfelt, A. Breier, S. A. Rubin, and K. M. Carbone.** 1997. Borna disease virus antibodies and the deficit syndrome of schizophrenia. Schizophr. Res. 23:253–257.
43. **Weisman, Y., D. Huminer, M. Malkinson, R. Meir, S. Kliche, W. I. Lipkin, and S. Pitlik.** 1994. Borna disease virus antibodies among workers exposed to infected ostriches. Lancet 344:1232–1233.



Short Contents | Full Contents Other books @ NCBI

Navigation

About this book

Appendix I. Immunologists' Toolbox

Immunization.

The detection, measurement, and characterization of antibodies and their use as research and diagnostic tools.

Isolation of lymphocytes.

Characterization of lymphocyte specificity, frequency, and function.

Detection of immunity *in vivo*.

Manipulation of the immune system.

Search

Go

This book All books

PubMed

Immunobiology ➔ **Appendix I. Immunologists' Toolbox** ➔ Isolation of lymphocytes.

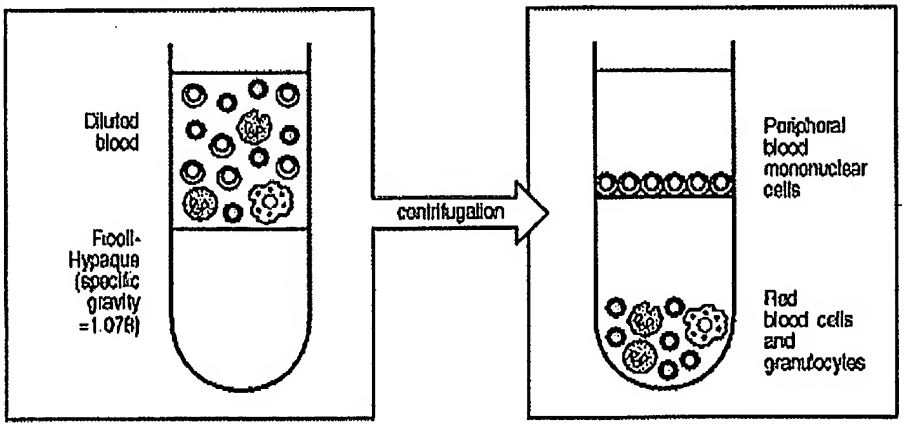


Figure A.23. Peripheral blood mononuclear cells can be isolated from whole blood by Ficoll-Hypaque™ centrifugation. Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque™ and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are more dense and centrifuge through the Ficoll-Hypaque™, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface (right panel).

A9

ANNALS OF SURGERY
Vol. 226, No. 1, 43–50

Prospective Evaluation of Circulating Hepatocytes by Alpha-Fetoprotein mRNA in Humans During Liver Surgery

Antoinette Lemoine, Ph.D.,* Thierry Le Bricon, Ph.D.,* Marina Salvucci, Pharm.D.,* Daniel Azoulay, M.D.,† Patrick Pham, Pharm.D.,* Joseph Raccuia, M.D.,‡ Henri Bismuth, M.D., FACS(Hon),† and Brigitte Debuire, Ph.D.*

From the Service de Biochmie et Biologie Moléculaire and the Centre Hépatobiliaire†— Hôpital Paul Brousse, Villejuif Cedex, France; Department of Surgery,‡ St. Vincent's Hospital, New York, New York.*

Objective
The objective of this study was to analyze the specificity of detecting liver tumor cell dissemination by alpha-fetoprotein (AFP) mRNA in peripheral blood.

Summary Background Data
Alpha-fetoprotein mRNA has been used for the detection of circulating micrometastatic tumor foci of hepatocellular carcinoma (HCC); however, the interpretation of the results has been equivocal.

Methods
Sixty-four consecutive patients with malignant HCC (n = 20), liver metastases (n = 27), or nonmalignant (n = 17) liver diseases undergoing partial or total hepatectomy and orthotopic liver transplantation were included in this prospective study from January to July 1995. Peripheral blood samples were obtained before surgery, during surgery, and after surgery (range, 6–15 months). Total mRNA was extracted from nucleated cells, and cDNA synthesis and polymerase chain reaction amplification (nested polymerase chain reaction in one tube) were performed with specific AFP primers.

Results
Preoperative AFP mRNA was detected in 20 patients (17%), of which 5 of 20 had HCC. Intraoperative assessment showed positive AFP mRNA values in a total of 34 patients (53%) with various causes, of which 8 of 20 (40%) had HCC, 17 of 27 (63%) had other malignancies, and 9 of 17 (53%) had nonmalignant diseases. Recurrent tumor in patients with HCC occurred in four cases after surgery (range, 6–15 months) and did not correlate with AFP mRNA positivity before surgery, during surgery, or after surgery.

Conclusions
Alpha-fetoprotein mRNA in peripheral blood is not a specific marker of circulating micrometastases from HCC, especially in the context of surgical treatment of HCC.

The result with both orthotopic liver transplantation (OLT) and hepatic resection with the intent to cure patients with hepatocellular carcinoma (HCC) has been dismal because of the high rate of recurrence despite thorough preoperative screening for extrahepatic disease.[1,2] This high incidence of recurrence indicates that malignant hepatocytes had entered the circulation either before or during surgery, therefore, arguing for some type of adjuvant chemotherapy when surgery is required. Detection of small numbers of circulating cells and examination of tumor markers have advanced with the refinement of molecular biology techniques. These advances have been popularized more recently with attempts to detect circulating tumor cells before dissemination is clinically apparent.[3-10] Two markers for circulating tumoral liver cells have been proposed. One involves the albumin mRNA[11-13] and, more recently, the other involves the presence of AFP mRNA in peripheral blood, which has been reported to be more specific in patients with HCC.[14-18] Particularly, these markers have been positive in patients with a high grade of HCC or with extrahepatic metastases.[15,18] However, the accuracy of this "HCC-specific" gene transcript has yet to be determined.

Equivocal interpretations from the previous studies concerning markers for circulating HCC cells prompted our group to initiate a prospective study of AFP mRNA specificity in a large group of consecutive patients undergoing either partial or total hepatectomy and OLT for various tumoral and nontumoral liver diseases. Blood samples were taken before and at two different timepoints during surgery (during the exploratory phase and after hepatectomy) to elucidate the status of AFP mRNA in the circulation of patients with malignant or nonmalignant liver disease during the course of liver surgery. We hypothesized that liver surgery would be associated with a release of hepatocytes into the bloodstream that could be detected using AFP mRNA as the target gene transcript, therefore, questioning its potential for clinical use regarding therapeutic decisions such as intraoperative chemotherapy or in the immediate postoperative period.

PATIENTS AND METHODS

Study Population

Sixty-four consecutive patients with malignant or nonmalignant liver diseases undergoing partial (n = 45) or total hepatectomy and OLT (n = 19) were included in this prospective study from January to July 1995. There were 20 patients with HCC (19 men and 1 woman), 2 with cholangiocarcinoma, 25 with liver metastases (12 men and 13 women) from colorectal carcinoma (n = 19), breast cancer (n = 3), and other types of malignancy (n = 3). Thirteen patients without liver tumor confirmed by histologic analysis had cirrhosis (11 men and 2 women), caused by alcoholism in 8 patients, chronic viral hepatitis C infection in 1, alcoholic disease associated with viral hepatitis C in 2, primary biliary cirrhosis in 1, and cirrhosis from unknown origin in 1. Four patients had neither liver tumor nor cirrhosis but two cases of amyloid neuropathy, one case of porphyria, and one case of focal nodular hyperplasia. Diagnosis of HCC was made by ultrasonography or computed tomography, serum AFP, and confirmed by final pathology results after surgical resection. Size (*i.e.*, maximal diameter of tumor), number of nodules in the liver, and total volume of the tumor were calculated using imaging and volumetric scanning techniques, including intraoperative ultrasound. Final pathology results determined the grade of HCC according to Edmonson classification, the presence of portal vein invasion, and the Child score for cirrhosis. The control group included 28 normal, healthy volunteers without liver diseases (14 men, 14 women; mean age, 30 years).

The majority of patients with HCC (15/20, 75%) received neoadjuvant chemoembolization, and serum AFP levels were normal (<20 ng/mL) at the time of the hepatectomy in 12 (60%) of 20 patients. Fifteen (56%) of the 27 patients operated on for liver metastasis from other cancers received neoadjuvant chronomodulated chemotherapy according to our institutional protocol.[19]

Alpha-Fetoprotein mRNA Assay

Peripheral blood samples were obtained before surgery and during surgery at two different intervals: the first during the exploratory phase and the second after hepatectomy was completed, using ethylenediaminetetraacetic acid as an anticoagulant. All patients surviving the early postoperative period (n = 59) were observed 6 to 15 months after hepatectomy with blood samples obtained during routine clinic examination. Sensitivity of our assay was determined with human hepatocytes isolated from cadaveric multiple organ donors.[20]

Nucleated cells were isolated from peripheral blood using tetradecyltrimethylammonium bromide as described elsewhere,[21] and total RNA was extracted from the pellet or from cryopreserved liver tissues according to our technique reported previously.[22] Special attention was given to the choice of AFP primers to avoid cross-reaction between albumin and AFP mRNA because they present a 50% homology at the mRNA level. The primers

Supported by grants from the Faculté de Médecine Paris-Sud and the Association pour la Recherche sur le Cancer (ARC).

Address reprint requests to Brigitte Debuire, Ph.D., Service de Biochimie et Biologie Moléculaire, Hôpital Paul Brousse, 14 Avenue Paul Vaillant Couturier, 94804 Villejuif Cédex, France.

Accepted for publication May 30, 1996.

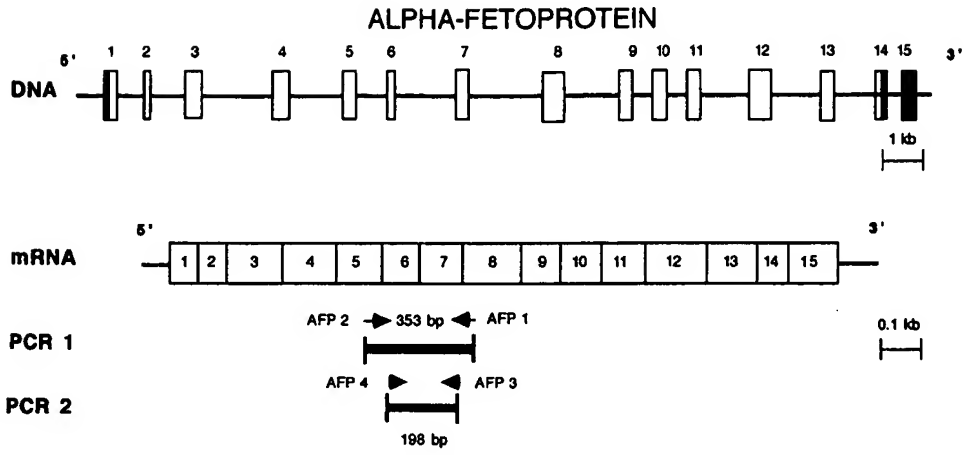


Figure 1. Representation of alpha-fetoprotein gene, alpha-fetoprotein gene transcript, and the position of primers used in reverse transcriptase–polymerase chain reaction.

were designed from the gene sequences of human serum alpha-fetoprotein.[23] To avoid false-positive results caused by DNA contamination, primers were selected in different exons of the AFP gene (Fig. 1). Sequences of primers used in the experiment were as follows. The sense primers were 5′ CAA TTC TTC TTT GGG CTG CTC GCT ATG AC 3′ (AFP 2) and 5′ ATG CAG TTG AAT GCT TCC AA 3′ (AFP 4), and the antisense primers were 5′ AGT GTC TTG TTG AGA ACA TAT GTA GGA CAT G 3′ (AFP 1) and 5′ CCA CAT CCA GGA CTA GTT TCT 3′ (AFP 3). The cDNA synthesis and polymerase chain reaction (PCR) amplification procedure (nested PCR in one tube) were performed as reported already by us.[22] Size of the amplified products of AFP mRNA was 198 base pairs.

Statistical Analysis

Statistical analysis was performed to determine if the influence of the indication for surgery (HCC *vs.* other indications), the surgical procedure itself (partial *vs.* total hepatectomy), and/or the use of neoadjuvant chemotherapy could affect detection of AFP mRNA in the circulation. The test results in the study group of HCC were correlated to the incidence of recurrence. Results are presented as mean ± standard deviation. Statistical analysis was performed with a statistical program (Statistica; StatSoft, Tulsa, OK) using the chi square test with a significance level set at 0.05.

RESULTS

Alpha-Fetoprotein mRNA Assay Sensitivity and Control Group

The sensitivity of our assay, determined in a dilution experiment (Fig. 2) using freshly isolated human hepatocytes (10^5 to 10^1) in 1 mL whole blood before RNA extraction, was approximately 1 hepatocyte for 10^5 peripheral mononuclear cells. Alpha-fetoprotein mRNA was not detected in the peripheral blood of 28 healthy subjects.

Preoperative Detection of Alpha-Fetoprotein mRNA

Before hepatectomy, AFP mRNA was detected in the blood of 11 (17%) of the 64 patients diagnosed with HCC (n = 5/20), cholangiocarcinoma (n = 1/2), colorectal carcinoma with liver metastasis (n = 4/25), and chronic active viral C hepatitis infection (n = 1/13). Patients with malignant disease and detectable AFP mRNA before hepatectomy (n = 10/47) had received chemotherapy, which was stopped 3 to 12 months before surgery (average, 6 months).

Intraoperative Detection of Alpha-Fetoprotein mRNA

During surgery, a total of 34 (53%) of 64 patients had detectable AFP mRNA at either 1 or both sampling inter-

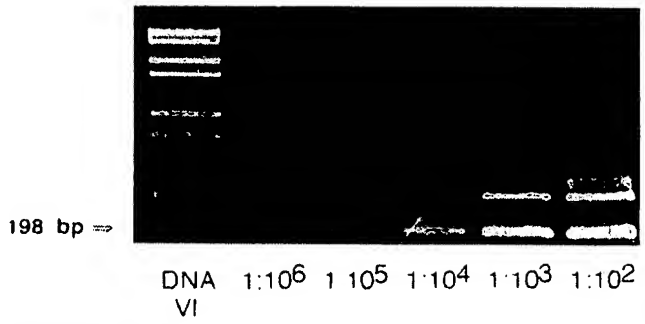


Figure 2. Sensitivity of the assay. Sensitivity of our assay was established using dilution of freshly isolated normal hepatocytes ($10,^5$ $10,^4$ $10,^3$ $10,^2$ 10) in normal blood before total RNA extraction.

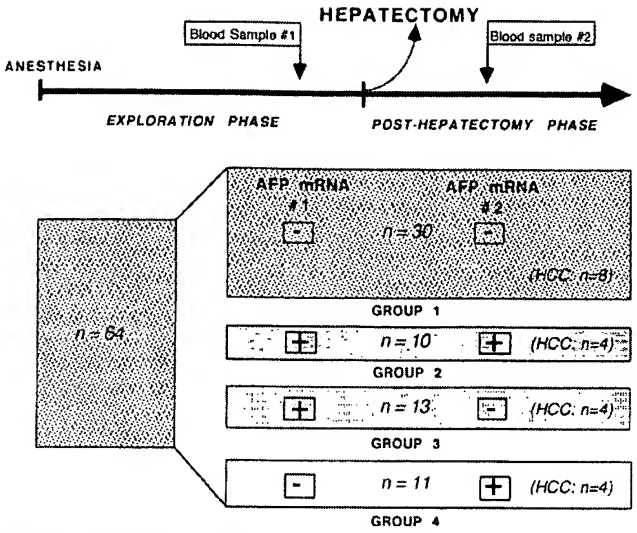


Figure 3. Schematic representation of intraoperative alpha-fetoprotein (AFP) mRNA profile in patients who are operated on. Group 1 = patients with no detectable AFP mRNA, group 2 = patients with detectable AFP mRNA at both blood samples, group 3 = patients with detectable AFP mRNA at the first blood sample only, group 4 = patients with detectable AFP mRNA at the second blood sample only.

vals. Alpha-fetoprotein mRNA was observed in 23 of the 64 patients during the exploration phase (first intraoperative blood sample), whereas 10 of these latter patients and 11 others were positive for AFP mRNA after hepatectomy (second intraoperative blood sample). Patients could be divided in four groups according to their intraoperative AFP mRNA profile (Fig. 3). In group 1, the largest (n = 30), patients had no detectable AFP mRNA during liver surgery, including 8 patients who received partial hepatectomy for HCC. In group 2 (n = 10, including 4 HCC), patients had detectable AFP mRNA at both the first and the second operative timepoints. In group 3 (n = 13, including 4 patients treated for HCC), patient results were positive at the first blood sampling and then negative in the second. Conversely, in group 4 (n = 11, including 4 patients treated for HCC), patient results were negative at the first blood sampling and positive after hepatectomy.

Correlations Between Alpha-Fetoprotein mRNA Test Results and Clinical Characteristics

The presence of detectable intraoperative AFP and mRNA was not influenced significantly either by the disease or by the type of surgical procedure (Table 1). In patients with malignant disease, the presence of detectable AFP mRNA did not correlate with the use of neoadjuvant chemotherapy (Table 1). The 20 patients with HCC included 4 patients receiving OLT and 16 having partial hepatectomy (Table 2). Forty percent of patients with HCC had more than one tumor foci, and the majority (n = 17, 85%) were encapsulated tumors. None of the tumors were of the fibrolamellar variety. Hepatocellular carcinoma was impossible to grade in 60% of patients because of tumor necrosis, whereas in the remaining patients, only three were classified as grade III or IV. Vascular invasion was present in 50% of the patients examined. No AFP mRNA was detected in 40% (n = 8) of patients who were operated for HCC (Table 2, Fig. 4). During surgery, in 40% of cases, AFP mRNA was detected in one of the two intraoperative samples, and four patients had detectable AFP mRNA at both sampling points. There was no evident relation between the presence of AFP mRNA in the blood during surgery and the serum level of the tumor marker AFP, the characteristics of the tumor (in particular the size and number of tumor foci), and the presence of vascular invasion.

Correlations Between Alpha-Fetoprotein mRNA Test Results and Clinical Outcome

Complete follow-up with peripheral blood sampling was performed 6 to 15 months after surgery. Eighteen (28%) of 64 patients had detectable AFP mRNA (1/17 nontumor causes, 7/27 secondary metastases, and 10/20 HCC) during the course of follow-up. The patient who had a signal for AFP mRNA without hepatic tumor had active chronic viral C hepatitis, whereas in the group of patients with metastatic tumor, only two of the four who had positive results before surgery remained with positive results 8 and 11 months after surgery, respectively. In the group of patients with HCC, three died in the immediate postoperative period, two from terminal liver failure and one from primary nonfunction after transplantation. These were three patients without detectable AFP mRNA during surgery (patient 20; Table 2) or only one positive test result during surgery (patients 16, 17; Table 2). The follow-up available for the 17 surviving patients ranges from 6 to 14 months (median, 9 months). Ten patients with HCC had positive results and seven had negative results for AFP mRNA signal after surgery. Among the five patients who had a signal for AFP mRNA before surgery, only one had a negative result 9 months after surgery (patient 1, Table 2). There were three patients without detectable AFP mRNA during surgery that remained with negative results (patients 1, 2, 11), whereas the results of three others became positive during surgery (patients 3, 12, 14). Two patients in the latter group had recurrent viral C hepatitis. In the group of patients that had detectable AFP mRNA during surgery, the results of seven remained positive (patients 4, 5, 6, 7, 10, 13, 18), whereas the results of four others became negative by subsequent testing (patients 8, 9, 15, 19). Finally, four patients[8-10,13]

Table 1. INFLUENCE OF INDICATION, TYPE OF SURGERY, AND PREOPERATIVE CHEMOTHERAPY ON INTRAOPERATIVE ALPHA-FETOPROTEIN mRNA IN PERIPHERAL BLOOD*

	Number of Patients (n = 64)	Alpha-Fetoprotein mRNA		
		Negative (n = 30)	Positive (n = 34)	
Indication				
Hepatocellular carcinoma	20	8 (40)	12 (60)	NS
Liver metastasis of other cancers	27	11 (41)	16 (59)	
Nonmalignant liver diseases	17	11 (65)	6 (35)	
Surgery				
Total hepatectomy and OLT	19	14 (74)	5 (26)	NS
Partial hepatectomy	45	17 (38)	28 (62)	
Chemotherapy				
Yes	28	10 (36)	18 (64)	NS
No	20	7 (35)	13 (65)	

OLT = orthotopic liver transplantation; NS = not significant.

* Patients were considered negative if no AFP mRNA was detected intraoperatively, positive if AFP and mRNA was detected in at least one of the two intraoperative samples. Chemotherapy was performed preoperatively in 28 of 47 patients with malignant liver disease. Statistical analysis was performed using the chi square test (significance set at $p < 0.05$). Values are number (%).

Table 2. HISTOLOGIC PARAMETERS OF PATIENTS OPERATED FOR HEPATOCELLULAR CARCINOMA

Patient Number	Preoperative AFP (ng/mL)	Chemoembolization	Number (size, cm) of Tumors*	Capsule	Grade†	Liver	Vascular Invasion	AFP mRNA‡				Recurrence
								Pre	1	2	Post	
1	<20	Yes	>3 (15)	No	II	NC	Yes	+	–	–	–	No
2	<20	No	1 (20)	Yes	I	NC	Yes	–	–	–	–	No
3	<20	Yes	2 (9)	Yes	II	NC	Yes	–	–	–	+	No
4	<20	Yes	1 (5)	Yes	N	C	Yes	+	+	+	+	No
5	215	Yes	1 (5)	Yes	N	NC	No	–	+	–	+	No
6	<20	Yes	3 (5)	Yes	N	NC	No	+	+	–	+	No
7	<20	No	1 (1.5)	Yes	N	C	No	+	–	+	+	No
8	10,350	Yes	1 (6)	Yes	III or IV	C	No	–	–	+	–	Yes
9	694	Yes	1 (4.5)	Yes	N	C	No	–	–	+	–	Yes
10	<20	Yes	2 (4)	Ruptured	III	NC	Yes	–	+	–	+	Yes
11	99	Yes	>3 (14)	Yes	II	NC	Yes	–	–	–	–	No
12	<20	Yes	2 (4)	Yes	N	C	No	–	–	–	+	No
13	2056	Yes	2 (7)	Yes	III	NC	No	–	+	+	+	Yes
14	593	Yes	1 (15)	Yes	N	NC	Yes	–	–	–	+	No
15	<20	Yes	2 (3)	No	N	C	No	–	–	+	–	No
16	40	No	1 (3)	No	II	NC	Yes	–	+	–	ND	Died
17	<20	No	1 (2)	Yes	N	C	No	–	–	–	ND	Died
18	<20	Yes	1 (6)	Yes	N	C	Yes	+	+	+	+	No
19	<20	Yes	1 (8)	Yes	N	NC	Yes	–	+	+	–	No
20	<20	Yes	1 (10)	Yes	N	C	No	–	–	–	ND	Died

AFP = Alpha-fetoprotein; N = necrosis; NC = noncirrhotic; C = cirrhotic; ND = not done.

* For multiple tumors, the size of the tumor indicated in the parentheses is the size of the largest one.

† Grade of the tumor was determined according to Edmonson. None of the patients studied had metastases to lymph nodes or distant organs.

‡ AFP mRNA was detected during partial hepatectomy or orthotopic liver transplantation (patients 7, 10, 11, and 12) at two time points (exploration phase, 1; after hepatectomy, 2) and 6–15 months later.

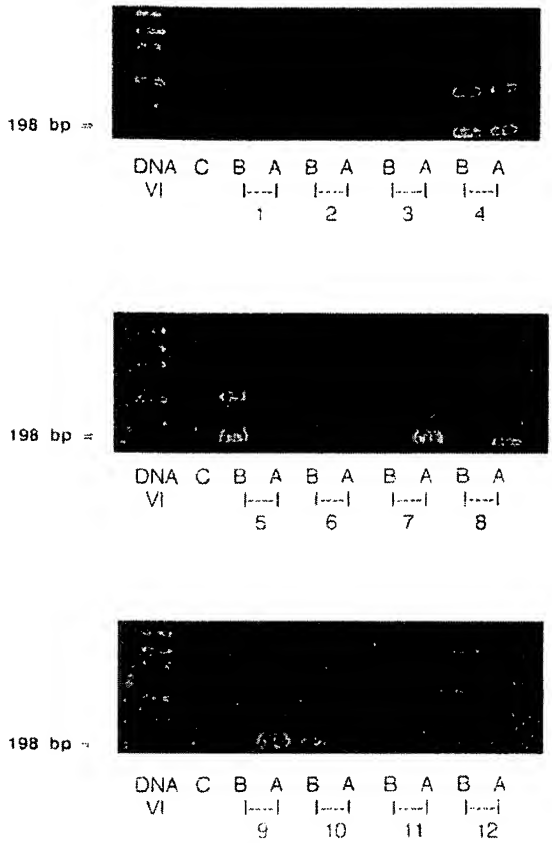


Figure 4. Electrophoresis pattern of 12 randomly chosen patients with hepatocellular carcinoma in our series of 20 cases. (A) After partial or total hepatectomy (second intraoperative blood sample) and (B) before partial or total hepatectomy (first intraoperative blood sample). Detailed characteristics of these patients are provided in Table 2. DNA VI = size marker, (C) negative control sample. Larger bands correspond to residual amplification products of the first polymerase chain reaction round.

had detectable recurrence by abdominal ultrasound, thoracoabdominal computed tomographic scan, bone scintiscan, or serum AFP levels. Of these four patients with recurrence, all had negative preoperative AFP mRNA values, but all had positive values shown during the intraoperative examination. In the immediate postoperative period, the test result was positive in only two of the patients with recurrent disease. There was no correlation between the incidence of recurrence and the test results before, during, and after surgery.

DISCUSSION

The results of this prospective study to evaluate AFP mRNA in peripheral blood lead us to conclude that this is not specific for circulating tumor foci. The use of this test, especially in the context of surgical treatment of HCC, cannot yet be recommended despite the suggestion of others in previous reports that AFP mRNA or albumin mRNA could be used clinically as a specific indicator of hematogenous dissemination of HCC cells.[11-18] We, as others,[14,15] examined albumin mRNA in the peripheral blood by PCR; however, its presence was shown in all control samples tested (n = 28, data not shown), whereas none of the control samples using AFP mRNA had tested positive. These results give credence to the concept of "illegitimate" transcription of the albumin gene in mononuclear cells.[14,15,24] Consequently, the detection of AFP gene transcripts seemed to be a valid method to identify abnormal liver cells in the circulation and, therefore, the possibility to visualize circulating HCC micrometastatic foci. None of our control patients had a positive signal for AFP mRNA, as also reported previously.[15]

Before surgery, we found a similar rate of detection in patients with HCC (25%) than in patients with other malignant liver diseases (20%). This is the first report documenting an association between detectable AFP mRNA in the circulation and liver metastases of other cancers. In the series of Komeda et al.,[18] none of the eight patients with metastatic liver cancer exhibited AFP mRNA in the blood, although another patient without cancer and chronic active viral C hepatitis had a signal for AFP mRNA before surgery. The presence of AFP mRNA already has been reported in cirrhosis (0%–15%) as well as acute and chronic hepatitis (0%–75%).[11,15-17] However, if only the preoperative test results of the nonmalignant patients (17 nonmalignant liver disease and 28 healthy control subjects) are considered, then only 1 of 45 was positive for AFP mRNA. The percentage of detectable AFP mRNA before surgery in patients with HCC (25%) was lower than that from previous results reported by others (36%–52%).[15,18] These differences cannot be explained by a lower sensitivity of our technique because a reverse transcription followed by nested PCR assay in one tube[25,26] is considered the most sensitive available (theoretically one abnormal cell for 10^6) with limited cross-contamination and is perfectly adapted to clinical laboratory use. In addition, when compared to methods involving preliminary isolation of cells,[12,15,17] our processing of blood samples is rapid, easy, and inexpensive. Furthermore, in our experience, techniques using Ficoll (Pharmacia, Uppsala, Sweden) cell extraction or dextran-based technique described by Matsumura et al.[16] obtained approximately ten times lower sensitivity than with tetradecyltrimethylammonium bromide (data not shown). Differences in AFP mRNA results in patients with HCC are more likely explained by the characteristics of patients examined, because in our report, only 3 patients (15%) had HCC grade III or IV, 12 other patients showed total tumor necrosis, thus rendering it impossible to grade, and no patient had known extrahepatic disease. This suggests that in these patients, few or no intact tumor cells would be circulating in the bloodstream. The fact that most patients

(75%) had received neoadjuvant arterial chemotherapy before surgery could explain why AFP serum levels were normal (<20 ng/mL) in many patients (65%). Although all of these factors individually might influence the potential for dissemination and subsequent detection of AFP mRNA, this has yet to be elucidated completely. In the series by Komeda et al.,[18] the presence of AFP mRNA in peripheral blood clearly was related to the stage of the disease (10% in stages I and II, 30% in stage III, and 77% in stage IV). However, the use of AFP mRNA as a marker of tumor dissemination would be useful if the tumor is at a stage when it is still considered curable, such as with stage I and II disease when AFP levels still are presumably at low levels in the group of patients examined in our series. The use of AFP mRNA in patients with late-stage (stage IV) disease seems elaborate considering that even if specific for metastases, this information most likely would be ascertained by more conventional clinical imaging methods. The subset of patients who theoretically would benefit would be at clinical stage I or II disease with positive AFP mRNA results, which then would alter the clinical therapeutic decision in the form of treatment rendered.

In our group of 64 consecutive patients undergoing either partial or total hepatectomy, detection of AFP mRNA in peripheral blood intraoperatively was possible in 53%, regardless of the disease. Detectable AFP mRNA could not be explained by the surgical indication (60% for HCC *vs.* 59% for liver metastases), and no significant differences were observed between partial or total hepatectomy (62% *vs.* 26%). In addition, the use of neoadjuvant chemotherapy (64% *vs.* 65%) and total or partial vascular exclusion of the liver during hepatectomy (data not shown) did not affect significantly the incidence of detecting AFP mRNA in peripheral blood. Our results suggest that liver manipulation clearly leads to hematogenous spread of cells from liver origin, which can be detected by AFP mRNA. The release of cancer cells has been shown during tumor resection by immunocytochemical techniques and PCR in breast cancer,[7] colorectal cancer,[27] and primary renal cancer.[28] Similarly, chemotherapy has been shown to induce a release of circulating AFP mRNA-positive cells in the circulation in small groups of patients with HCC.[18] Alpha-fetoprotein gene transcripts are found not only in HCC cells, but also in normal liver cells[11]; therefore, the malignant origin of the detected cells cannot be established or ruled out. Surprisingly, AFP mRNA signal fluctuates rapidly during the course of liver surgery. For example, patients in group 3 have a release of hepatocytes at the time of the first blood sample but not at the second endpoint, theoretically the most traumatic period. These results concur with other experimental[25,26] and clinical data[27,28] suggesting that release of abnormal cells in the circulation, either spontaneously or secondary to surgical manipulation, is an intermittent and transient phenomenon. As a result, detection of AFP mRNA might instead be dependent on the timing of the peripheral blood sampling, which further indicates the high likelihood of a "sampling error" during surgery.

During follow-up, 17 (27%) of 64 had positive AFP mRNA signals, of which 3 had chronic active viral C hepatitis. Follow-up results of patients operated on for breast cancer[7] or after chemotherapy for HCC[18] have shown that previously positive samples failed to show target gene expression 24 hours and 7 days after treatments, respectively. Analysis of HCC in our series shows ten with positive signals, whereas three had negative signals intraoperatively. Subsequently, only four patients with HCC had recurrent disease diagnosed; however, only two of these patients tested positive for AFP mRNA. The sensitivity and specificity of this test for clinical decisions, especially regarding therapy, were low (50% and 36%, respectively) with a predictive value of only 18%. This is contrary to previous studies because in only one report, disease recurrence occurred in three patients when albumin mRNA was measured in the peripheral blood of nine patients with HCC (stages II and IV) after OLT.[12] It cannot be overemphasized that reports of illegitimate transcription have been well documented with the use of albumin mRNA[14,15,24] since the test was described initially.[11,12] These results indicate that the malignant origin of detected circulating cells cannot be established by AFP mRNA that fluctuates over time, thus could represent a "sampling error." Therefore, the prognostic value of intraoperative circulating cancer cells shows both low sensibility and specificity.

The current study does reflect the inaccuracy of using the AFP mRNA as a biologic marker for detecting circulating tumor micrometastases in HCC and, therefore, the AFP gene cannot be considered an "HCC-specific" gene. Thus, suggesting different treatment options for patients with intraoperative detection of AFP mRNA cannot be advocated, and further studies are necessary to isolate certain genetic markers, which are specific for micrometastatic foci of HCC. Rather than the use of "tissue-specific" genes, potential approaches for the detection of micrometastases include the identification of "metastatic" genes, which are expressed only in metastatic cells such as the one coding for CD44, a glycosylated cell surface adhesion molecule, or the detection of an "HCC-specific" form of serum AFP. Until an accurate test is devised, it would be imprudent to attempt clinical application of AFP mRNA in the detection of micrometastatic foci.

Acknowledgments

The authors thank the staffs of the anesthesy unit and of the operating room.

References

1. Farmer DG, Rosove MH, Shaked A, Busuttil RW. Current treatment modalities for hepatocellular carcinoma. Ann Surg 1994; 219:236–247.
2. Okuda K. Hepatocellular carcinoma: recent progress. Hepatology 1992; 15:948–963.
3. Israeli RS, Miller WH, Su SL, et al. Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. Cancer Res 1994; 54:6306–6310.
4. Moreno JG, Groce CM, Fischer R, et al. Detection of hematogenous micrometastasis in patients with prostate cancer. Cancer Res 1992; 52:6110–6112.
5. Leather AJM, Gallegos NC, Kocjan G, et al. Detection and numeration of circulating tumor cells in colorectal cancer. Br J Surg 1993; 80:777–780.
6. Glaves D, Huben RP, Weis L. Haematogenous dissemination of cells from human renal adenocarcinomas. Br J Cancer 1988; 57:32–35.
7. Brown DC, Purushotham AD, Birnie GD, Georges WD. Detection of intraoperative tumor cell dissemination in patients with breast cancer by use of reverse transcription and polymerase chain reaction. Surgery 1995; 117:96–101.
8. Smith B, Selby P, Southgate J, et al. Detection of melanoma cells in peripheral blood by means of polymerase chain reaction. Lancet 1991; 338:1227–1229.
9. Naito H, Kuzumaki N, Uchino J, et al. Detection of tyrosine hydroxylase mRNA and minimal neuroblastoma cells by the reverse transcription-polymerase chain reaction. Eur J Cancer 1991; 27:762–765.
10. Carr BI, Kar S. An assay for hepatoma micrometastasis: albumin gene expression in peripheral blood from patients with advanced hepatocellular carcinoma (HCC). Hepatology 1991; 14:108A.
11. Hillaire S, Barbu V, Boucher E, et al. Albumin messenger RNA as a marker of circulating hepatocytes in hepatocellular carcinoma. Gastroenterology 1994; 106:239–242.
12. Kar S, Carr B. Detection of liver cells in peripheral blood of patients with advanced-stage hepatocellular carcinoma. Hepatology 1995; 21:403–407.
13. Carr BI. An assay for micrometastatic hepatocellular carcinoma. Gastroenterology 1994; 106:264–267.
14. Chou HC, Sheu JC, Huang GT, et al. Albumin messenger RNA is not specific for circulating hepatoma cells. Gastroenterology 1994; 107:630–631.
15. Matsumara M, Niwa Y, Kato N, et al. Detection of alpha-fetoprotein mRNA, an indicator of hematogenous spreading hepatocellular carcinoma, in the circulation: a possible predictor of metastatic hepatocellular carcinoma. Hepatology 1994; 20:1418–1425.
16. Matsumura M, Niwa Y, Hikiba Y, et al. Sensitive assay for detection of hepatocellular carcinoma associated gene transcription (alpha-fetoprotein mRNA) in blood. Biochem Biophys Res Comm 1995; 207:813–818.
17. Nambu S, Nishimori H, Saeki M, et al. Alpha-fetoprotein messenger RNA in peripheral blood as a marker of circulating hepatocellular carcinoma cells. Int Hep Comm 1995; 3:217–221.
18. Komeda T, Fukuda Y, Sando T, et al. Sensitive detection of circulating hepatocellular carcinoma cells in peripheral venous blood. Cancer 1995; 75:2214–2219.
19. Levi F, Zidani R, Vannetzel JM, et al. Chronomodulated versus fixed-infusion-rate delivery of ambulatory chemotherapy with oxaliplatin; fluorouracil, and folinic acid (Leucovorin) in patients with colorectal cancer metastases: a randomized multi-institutional trial. J Natl Cancer Inst 1994; 26:1608–1616.
20. Pichard L, Fabre I, Daujat M, et al. Effect of corticosteroids on the expression of cytochromes P450 and on cyclosporin A oxidase activity in primary cultures of human hepatocytes. Mol Pharmacol 1992; 41:1047–1055.
21. MacFarlane DE, Dahle CE. Isolating RNA from whole blood. The dawn of RNA-based diagnosis. Nature 1993; 362:186–188.
22. Frenoy N, Chabli A, Sol O, et al. Application of a new protocol for nested PCR to the detection of minimal residual bcr/abl transcripts. Leukemia 1994; 8:1411–1414.
23. Gibbs PEM, Zielinski R, Boyd C, Dugaiscyk A. Structure, polymorphism, and novel repeated DNA elements revealed by a complete sequence of the human alpha-fetoprotein gene. Biochemistry 1987; 26:1332–1343.
24. Chelly J, Concordet JP, Kaplan JC, Kahn A. Illegitimate transcription: transcription of any gene in any cell type. Proc Natl Acad Sci U S A 1989; 86:2617–2621.
25. Mayew E, Glaves D. Quantification of the tumorigenic dissemination of arrested cancer cells. Br J Cancer 1984; 50:159–166.
26. Fidler IJ. Metastasis: quantitative analysis of distribution and fate of tumor cell emboli labeled with ^{125}I-5-iodo-2-deoxyurinide. J Natl Cancer Inst 1970; 45:773–782.
27. Leather AJM, Gallegos NC, Kocjan G, et al. Detection and numeration of circulating tumor cells in colorectal cancer. Br J Surg 1993; 80:777–780.
28. Glaves D, Huben RP, Weis L. Haematogenous dissemination of cells from human renal adenocarcinomas. Br J Cancer 1988; 57:32–35.

A10

Liu Y *et al.*, 1998. [The detection of circulating hepatocellular carcinoma cells in peripheral venous blood by reverse transcription-polymerase chain reaction and its clinical significance]. Zhonghua Wai Ke Za Zhi. 36:608-10. [Article in Chinese]

OBJECTIVE: To detect circulating hepatocellular carcinoma by demonstrating hepatocellular carcinoma cells-associated mRNA in the nuclear cell component of peripheral blood (PBL). METHOD: Peripheral blood (5 ml) samples were obtained from 93 patients with hepatocellular carcinoma (HCC) and from 37 controls (15 controls with liver cirrhosis after hepatitis B, 12 chronic hepatitis B, and 10 normal liver function). To identify HCC cells in peripheral blood, liver-specific human alpha-fetoprotein (AFP) mRNA was amplified from total RNA extracted from whole blood by reverse transcription-polymerase chain reaction. RESULT: AFP mRNA was detected in 50 blood samples from the patients with HCC (53.8%). In contrast, there were no clinical control patients whose samples showed detectable AFP mRNA in PBL. The presence of AFP mRNA in blood seemed to be correlated with the stage (by TNM classification) of HCC, the serum AFP value, and the presence of intrahepatic metastasis, portal vein thrombosis, tumor diameter and/or distant metastasis. AFP mRNA was detected in the blood of 21 patients showing metastasis at extrahepatic organs (100%) in contrast to 29 of 72 patients without metastasis (40.3%). CONCLUSION: The presence of AFP mRNA in peripheral blood may be an indicator of malignant hepatocytes, which might predict hematogenous spreading metastasis of tumor cells in patients with HCC.

PMID: 11825477 [PubMed - indexed for MEDLINE]

A 11

Detection of α-Fetoprotein mRNA, an Indicator of Hematogenous Spreading Hepatocellular Carcinoma, in the Circulation: A Possible Predictor of Metastatic Hepatocellular Carcinoma

MASAYUKI MATSUMURA,[1] YASURO NIWA,[1] NAOYA KATO,[1] YUTAKA KOMATSU,[1] SHUICHIRO SHIINA,[1] TAKAO KAWABE,[1] TAKEO KAWASE,[1] HIROSHI TOYOSHIMA,[2] MASASHI IHORI,[2] YASUSHI SHIRATORI[1] AND MASAO OMATA[1]

[1]*Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, and* [2]*Department of Gastroenterology and Hepatology, Japanese Red Cross Medical Center, 4-1-22 Hiroo, Shibuya-ku, Tokyo 150, Japan*

We attempted to detect circulating hepatocellular carcinoma by demonstrating hepatocyte-associated mRNA in the nuclear cell component of peripheral blood using nested reverse transcription–polymerase chain reaction because of the extremely small number of tumor cells in the circulation. Albumin mRNA was demonstrated not only in the liver tissue (hepatocytes) and HepG2 cells but also in nuclear cells of the blood from normal healthy volunteers (neutrophils and lymphocytes) by reverse transcription–polymerase chain reaction. In contrast, α-fetoprotein mRNA was demonstrated in the liver tissue, as well as in HepG2 cells, but not in peripheral blood of normal healthy volunteers, indicating the possibility of using α-fetoprotein mRNA for detection of benign and malignant hepatocytes among the population of neutrophils and lymphocytes. α-Fetoprotein mRNA in peripheral blood was detected in 17 of 33 cases of hepatocellular carcinoma (52%), 2 of 13 cases of cirrhosis (15%) and 2 of 17 cases of chronic hepatitis (12%). α-Fetoprotein mRNA was not demonstrated in 26 cases of normal healthy volunteers (0%). Among the patients with hepatocellular carcinoma, total volume of tumor tissue, maximum size of tumor and serum α-fetoprotein level were markedly increased in the patients with α-fetoprotein mRNA in blood. In addition, α-fetoprotein mRNA was detected in the blood of all 6 patients showing metastasis at extrahepatic organs (100%), in contrast to 11 of 27 cases without metastasis (41%). From these results, we conclude that the presence of α-fetoprotein mRNA in peripheral blood may be an indicator of circulating malignant or benign hepatocytes, which might predict hematogenous spreading metastasis of tumor cells in patients with hepatocellular carcinoma. (HEPATOLOGY 1994;20:1418-1425.)

HCC often develops in patients with chronic hepatitis and cirrhosis in association with HCV or HBV infection. The number of patients with HCC in association with HCV infection has increased recently, and the incidence of HCC among patients with cirrhosis is more than 6%/yr (1).

HCC is detected with several imaging techniques such as ultrasonography, abdominal angiography and computed tomography. HCC patients undergo medical and surgical treatments such as resection of the liver lobule (2), transarterial embolization (3) and percutaneous ethanol injection therapy (4). However, intrahepatic or extrahepatic metastasis to lung, bone and adrenal glands is frequently found; this is an indicator of poor prognosis in these patients (5). In recent years, liver transplantation has frequently been performed in patients with cirrhosis at an advanced stage, but these patients sometimes have HCC. However, extrahepatic metastasis of HCC reduces the beneficial effects of liver transplantation (6). Therefore early detection of micrometastasis of HCC could be a tool useful in selection of patients with HCC for liver transplantation. However, it is very difficult to demonstrate circulating malignant cells in blood morphologically; the number of malignant cells in the circulation is extremely small.

Recent studies have reported that tumor-specific genes are used to detect tumor cells in the circulation (7, 8). In relation to the biology of hepatocytes and HCC, these cells are known to produce albumin, AFP or both (9, 10). Therefore the detection of these hepatocyte-associated mRNAs in nuclear cell components of pe-

Received November 18, 1993; accepted June 1, 1994.

Other abbreviations used in the text: AFP, α-fetoprotein; DEPC, diethylpyrocarbonate; EDTA, ethylenediaminetetraacetate; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RT-PCR, reverse transcription–polymerase chain reaction.

This work was supported by grants from Ministry of Education, Culture and Sciences of Japan.

Address reprint requests to: Masayuki Matsumura, M.D., Second Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

0270-9139/94 $3.00 + 0 31/1/59373

TABLE 1. Patient profiles

Characteristics	HCC	Liver cirrhosis	Chronic hepatitis	Control
No. of patients	33	13	17	26
Age (yr)[a]	63 ± 9 (33-84)	65 ± 8 (45-75)	47 ± 12 (27-61)	57 ± 16 (33-83)
Sex (M/F)	26:7	7:6	15:2	18:8
Viral serological markers				
HBs Ag positive	6	2	0	0
HCV Ab positive	24	7	17	–
HBs Ag, HCV antibody negative	3	4	0	26
Child classification				
A	3	6	–	–
B	18	4	–	–
C	12	3	–	–

[a]Data expressed as mean ± S.D. (range).

ripheral blood may indicate the presence of circulating malignant or benign hepatocytes.

Human albumin (11) and AFP (12) genes, consisting of 14 exons tandemly located at the fourth chromosome, are 19 and 17 kb long, respectively. The expression of these genes is regulated by variable mechanisms such as colloid osmotic pressure through the modulation of the promoter activities of these genes (13). AFP, an oncofetal protein, is synthesized mainly in hepatocytes of fetal liver and yolk sac, and AFP production by hepatocytes is rapidly reduced from the time of birth in inverse proportion to an increased synthesis of albumin (10, 14). AFP mRNA has lately been detected in tumor tissue of HCC but not in nontumorous tissue, and its gene expression is reported to be modulated by hypomethylation of the AFP gene (15). Hitherto, increased serum AFP concentration, a tumor marker in patients with HCC, is used in screening for HCC (16), but it cannot predict metastasis of HCC. The presence of circulating HCC cells, which may be released from tumor foci into the circulation, could be an indicator of hematogenous spread of tumor cells leading to extrahepatic metastasis of HCC.

In this study, in an attempt to demonstrate the possibility of hematogenous metastasis of HCC, we tried to detect circulating HCC, which might be released from tumor foci into the circulation, by detecting the hepatocyte-associated mRNA (albumin or AFP mRNA) in the nuclear cell component of peripheral blood using nested RT-PCR.

PATIENTS AND METHODS

Patients. Thirty-three patients with HCC (26 men and 7 women), 13 cases of cirrhosis (7 men and 6 women) and 17 cases of chronic hepatitis (15 men and 2 women), admitted at our hospital between September 1992 and June 1993, were examined. As controls, 26 age-matched normal, healthy volunteers without liver diseases (18 men and 8 women) were selected. HBsAg and HCV antibody in serum were assayed with the Reversecell kit (Yamanouchi Pharmaceutical Company Ltd., Tokyo, Japan) and with the HCV PHA Dainabot kit (Dainabot, Tokyo, Japan), respectively. As indicated in Table 1, HCV antibody was found in more than 50% of the patients with chronic hepatitis, cirrhosis and HCC, but HBsAg was detected in fewer than 20%. Diagnosis of HCC was made by ultrasonography or computed tomography and confirmed by histology of tumor tissue. Size (maximum diameter of tumor), the number of tumor foci in the liver and total volume of tumor tissue were calculated with several imaging techniques. Diagnosis of cirrhosis and chronic hepatitis was made on the basis of liver histology, as well as clinical data. Blood was collected from a peripheral vein in a disposable syringe containing 0.1% EDTA before transarterial embolization, percutaneous ethanol injection therapy or both.

In addition, blood was collected from the hepatic vein, aorta and inferior vena cava during cardiac catheterization in 15 patients with ischemic heart diseases. Among these patients, one had cirrhosis, one had chronic hepatitis in association with HCV infection and two had fatty liver (slightly increased AST/ALT concentrations in serum).

Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research review committee.

Preparation of Nuclear Cells from Peripheral Blood. Five milliliters of blood was mixed with 1 ml of 5% dextran–saline solution and left to set for 30 min at room temperature to yield erythrocyte sediment (17). Supernatant was collected and centrifuged at 500 *g* for 25 min. The cells were then mixed with autoclaved, distilled water for lysis of residual erythrocytes. We then restored isotonicity by adding the same volume of 1.8% NaCl solution after 25 sec. After centrifugation at 350 *g*, the cells were immediately frozen with liquid nitrogen and stored at −80° C until use.

Liver Specimens and Cell Line of Hepatocytes. Normal liver tissue was obtained from autopsied liver. HepG2 cell line (a gift from Dr. Makoto Noda in Riken Cell Bank; RCB459) was maintained in Dulbecco's minimal essential medium (Gibco-BRL, Grand Island, NY) containing 10% fetal calf serum (Gibco-BRL).

Extraction of RNA. RNA was extracted from blood nuclear cells, liver tissue and HepG2 cells by use of RNAzol B (Biotecx Laboratories, Houston, TX) according to the manufacturer's protocol. RNA was then dissolved in DEPC-treated water and stored at −80° C until use.

Synthesis of cDNA. One microgram of RNA, which was heated at 95° C for 5 min and cooled rapidly on ice, was diluted at a volume of 10 μl containing 50 mmol/L Tris-HCl (pH 8.3), 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 10 mmol/L dithiothreitol, 0.25 μg hexamer random primer (Takara Biochemicals, Kyoto, Japan), 18 units of RNAse inhibitor (Takara Biochemicals) and 200 units of M-MLV reverse transcriptase (Gibco-BRL). cDNA was synthesized by means of incubation at 42° C for 60 min. It

TABLE 2. Detection of AFP mRNA in nuclear cells of peripheral blood

Group	No.	AFP mRNA in blood		
		Positive	Negative	% Positive
HCC				
TOTAL	33	17	16	52%[a]
HBs Ag positive	6	4	2	
HCV Ab positive	24	11	13	
HBs Ag, HCV antibody negative	3	2	1	
Cirrhosis				
TOTAL	13	2	11	15%
HBs Ag positive	2	1	1	
HCV Ab positive	7	0	7	
HBs Ag, HCV antibody negative	4	1	3	
Chronic hepatitis				
TOTAL	17	2	15	12%
HBs Ag positive	0	0	0	
HCV Ab positive	17	2	15	
HBs Ag, HCV antibody negative	0	0	0	
Healthy volunteers	26	0	26	0%

[a] $p < 0.05$ vs. other groups (χ^2 test).

was then heated at 95° C for 10 min for inactivation of reverse transcriptase, cooled rapidly and stored at −20° C until use for the first PCR.

Sequence of Primers Used in Nested PCR. Sequences of primers used in the experiment were as follows. The primers for albumin gene (11) were 5′-AGAAAGTACCCCAAGTGT-CAA-3′ (no. 9) (nucleotides 14997 to 15017), 5′-AGCTGCGA-AATCATCCATAAC-3′ (no. 10) (nucleotides 17019 to 16999) for outer primers and 5′-ACTATCTATCCGTGGTCCTGA-3′ (no. N1) (nucleotides 15111 to 15115, 15534 to 15549), 5′-TCTTGATTTGTCTCTCCTTCT-3′ (no. N2) (nucleotides 15750 to 15730) for inner primers.

The primers for AFP gene (12) were 5′-CTCTTCCAG-CAAAGCACACTTC-3′ (no. 7) (nucleotides 15300 to 15320) and 5′-CTCTTCAGCAAAGCAGACTTC-3′ (no. 8) (nucleotides 18481 to 18461) for outer primers and 5′-GCTGACAT-TATTATCGGACAC-3′ (M1) (nucleotides 16985 to 17005) and 5′-AGCCTCAAGTTGTTCCTCTGT-3′ (M2) (nucleotides 18406 to 18386) for inner primers.

We checked integrity of blood RNA by amplifying β-globin mRNA (18) with primers 5′-ACCCAGAGGTTCTTTGAG-TC-3′ (no. 26) (nucleotides 295 to 314) and 5′-TCTGATAG-GCAGCCTGCACT-3′ (no. 27) (nucleotides 1426 to 1407).

The sense and antisense primers described above were selected from different exons so that we might distinguish amplification of RNA from contaminating DNA.

Nested PCR. Ten microliters of cDNA solution was mixed with 40 μl of the PCR reaction mixture containing 50 mmol/L Tris-HCl (pH 8.3), 44 mmol/L KCl, 1.1 mmol/L $MgCl_2$, 0.013% gelatin, 12.5 pmol of each primer (nos. 7 and 8, 9 and 10 and 26 and 27) and 1 unit *Taq* DNA polymerase (Ampli Taq; Takara Biochemicals). The reaction mixture was overlaid with 50 μl of mineral oil (Sigma Chemical Co., St. Louis, MO) and heated at 93° C for 5 min. It was subjected to a total of 25 cycles of heating at 93° C for 30 sec, 54° C for 45 sec and 72° C for 45 sec with a thermal cycler (GeneAmp PCR system 9600; Perkin-Elmer Cetus, Norwalk, CT). The reaction cycle was terminated by heating at 72° C for 7 min and cooling at 4° C. Five microliters of amplified product using albumin and AFP primer was then used in the second PCR.

Five microliters of the amplified sample was mixed with 5 μl of second PCR buffer (100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 10 mmol/L $MgCl_2$, 100 μg/ml gelatin), containing 12.5 pmol of each inner primer (M1 and M2, N1 and N2) and 1 unit of *Taq* DNA polymerase. It was then diluted to a volume of 50 μl with distilled water. The reaction cycle was the same as that used in the PCR, except that annealing temperature was 52° C when N1 and N2 were used as primers.

The PCR products were subjected to electrophoresis on 3% agarose gels and stained with ethidium bromide. The amplified products of albumin, AFP and β-globin were 222, 282 and 283 bp, respectively.

Statistics. Each value is expressed as the mean ± S.E.M. unless otherwise stated. Statistical analysis was performed with the Mann-Whitney U test or the χ^2 test. A p value less than 0.05 was considered significant.

RESULTS

Demonstration of Albumin and AFP mRNA in Liver Tissue and Peripheral Blood. As indicated in Figure 1, albumin mRNA was demonstrated not only in liver tissue and HepG2 cells but also in blood nuclear cells isolated from normal healthy volunteers. In contrast, AFP mRNA was detected in liver tissue and HepG2 cells but not in blood nuclear cells of healthy volunteers.

Detection of Albumin and AFP mRNA in Nuclear Cells of Peripheral Blood from Patients with Liver Diseases. Albumin mRNA was demonstrated in the blood of all normal, healthy volunteers and all patients with liver diseases. In contrast, as indicated in Figure 2 and Table 2, AFP mRNA in peripheral blood was detected in 17 of 33 cases of HCC (52%), 2 of 13 cases of cirrhosis (15%) and 2 of 17 cases of chronic hepatitis (12%) but not in any of 26 healthy volunteers. The detection rate for AFP mRNA in blood was significantly increased in patients with HCC compared with that in patients with cirrhosis or chronic hepatitis, or with normal healthy volunteers (each, $p < 0.05$). However, the detection rate for AFP mRNA in blood was not significantly different among the HCC patients with different serological viral markers.

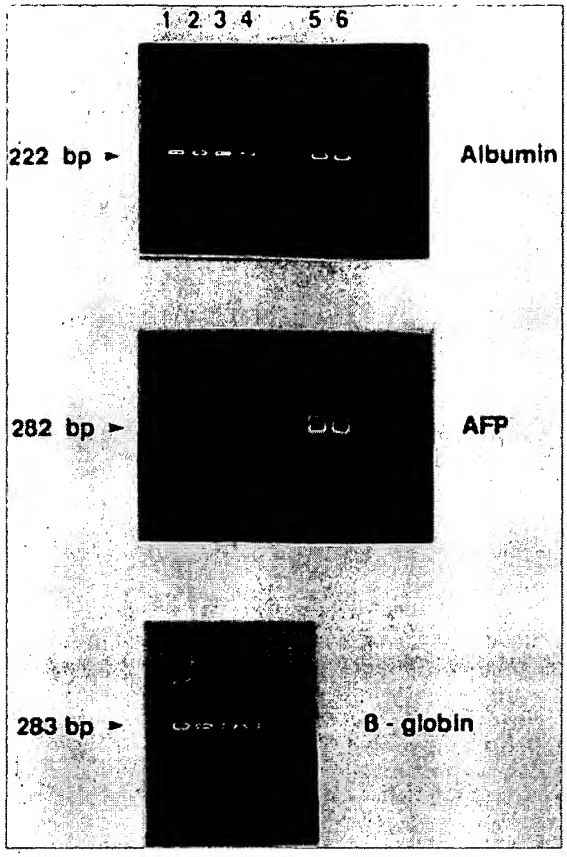


FIG. 1. Albumin and AFP mRNA in nuclear cells of peripheral blood, liver tissue and HepG2 cells as detected with nested RT-PCR. Albumin mRNA was detected in blood, as well as in liver tissue and HepG2 cells, whereas AFP mRNA was detected in liver tissue and HepG2 cells but not in blood. Demonstration of β-globin was performed as a control of RT-PCR. *Lanes 1 to 4:* nuclear cells of peripheral blood *(PBNC)* from healthy volunteers; *lane 5:* liver tissue; *lane 6:* HepG2 cells.

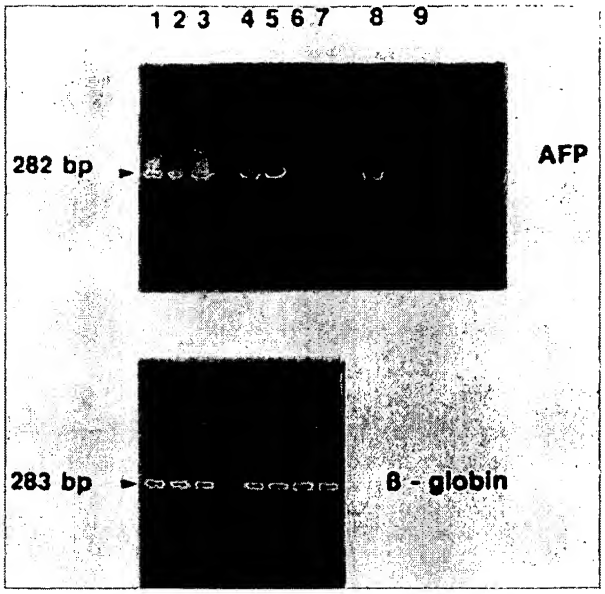


FIG. 2. Demonstration of AFP mRNA in blood of patients with HCC. *Lanes 1 to 3:* HCC with extrahepatic metastasis; *lanes 4 to 7:* HCC without extrahepatic metastasis; *lane 8:* liver tissue (positive control); *lane 9:* negative control of nested RT-PCR (DEPC-treated water was added to sample instead of RNA).

Detection of Albumin and AFP mRNA in the Nuclear Cells of the Blood Collected at Hepatic Vein, Aorta and Inferior Vena Cava. During cardiac catheterization in 15 patients with ischemic heart disease, blood was collected at the different sites. Albumin mRNA was demonstrated in nuclear cells of all the blood collected at different sites. However, as indicated in Table 3, AFP mRNA was detected in five cases at the hepatic vein and in one case at the inferior vena cava. Among these 15 patients, 1 was a patient with cirrhosis, 1 had chronic hepatitis in association with HCV infection and 2 had fatty liver. Among these 4 cases of liver disease, three had AFP mRNA in blood at the hepatic vein, and one patient with cirrhosis only had AFP mRNA in blood from the inferior vena cava. On the other hand, AFP mRNA in blood at hepatic vein was detected in only 2 cases of 11 cases without abnormal liver function, but AFP mRNA was not demonstrated in the blood at aorta and inferior vena cava in these 11 cases.

Characteristics of the HCC Patients with AFP mRNA in Peripheral Blood. Characteristics of the hepatocellular carcinoma patients with AFP mRNA in peripheral blood were clarified in relation to tumor size, number of tumor foci, total volume of tumor tissue in the liver, serum AFP concentration and presence of extrahepatic metastases.

In the cases with several tumor foci in the liver, the largest tumor was noted. AFP mRNA in blood was detected in seven of eight cases (87%) of HCC showing diffuse or large tumors occupying both lobes of the liver. As indicated in Figure 3, tumor diameter in the liver was significantly increased in the HCC patients with AFP mRNA in blood (8.1 ± 4.2 cm in diameter), when compared with the patients without AFP mRNA in the circulation (3.6 ± 2.8 cm in diameter) ($p < 0.05$). When the patients were divided into two groups according to diameter of tumor foci, the incidence of AFP mRNA in blood was markedly increased in the patients with HCCs 5 cm or more in diameter (14 of 17 cases [82%]) compared with those less than 5 cm in diameter (3 of 16 cases [19%]) ($p < 0.05$; χ^2 test).

Although the number of tumor foci was not significantly different between the groups with AFP mRNA detected and undetected group (3.8 ± 2.3 vs. 3.3 ± 2.1 foci, respectively) (Fig. 4), AFP mRNA in blood was detected in 9 of 15 cases (60%) of HCCs showing four or more tumor foci in the liver. In contrast, AFP mRNA was detected in 8 of 18 cases (44%) with fewer than 4 tumor foci (Fig. 4). Although there is a tendency toward an increased detection rate of AFP mRNA in the peripheral blood of patients with four or more tumor foci, it is not significantly compared with cases with fewer than four tumor foci in the liver.

We calculated total volume of tumor tissue by adding

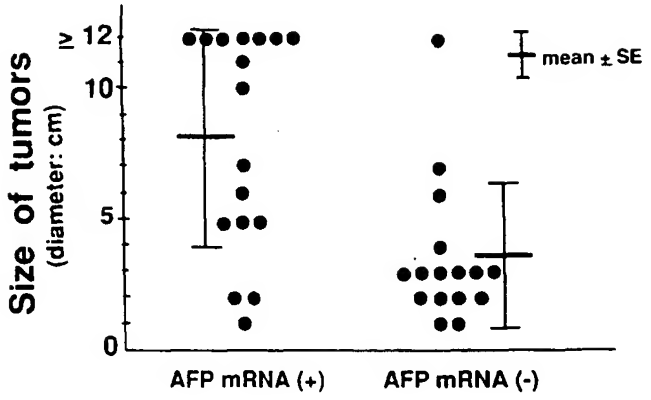


FIG. 3. Characteristics of patients with HCC in relation to size of tumor foci in the liver. Tumor size in the patients with AFP mRNA in blood was significantly larger than in those without AFP mRNA in blood ($p = 0.041$; Mann-Whitney U test).

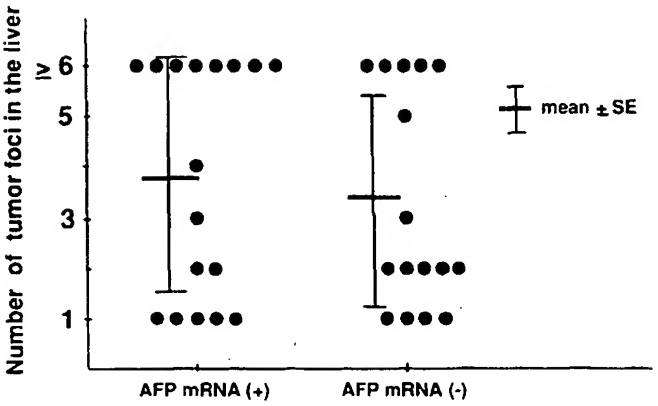


FIG. 4. Characteristic of patients with HCC in relation to the number of tumor foci in liver. There was a tendency toward an increased number of tumor foci in the patients with AFP mRNA in blood compared with that in patients without AFP mRNA, but it was not significant ($p = 0.584$, Mann-Whitney U test).

TABLE 3. Detection of AFP mRNA in blood collected at different sites

Patient no.	Hepatic vein	Aorta	Inferior vena cava	Liver disease
1	+	ND	+	Alcoholic cirrhosis
2	+	–	–	Chronic hepatitis (HCV antibody positive)
3	+	–	–	Fatty liver (ALT, 50 U/L)
4	–	–	–	Fatty liver (ALT, 45 U/L)
5	+	–	–	–
6	+	–	–	–
7	–	–	–	–
8	–	–	–	–
9	–	–	–	–
10	–	–	–	–
11	–	–	–	–
12	–	–	–	–
13	–	–	–	–
14	–	–	–	–
15	–	–	–	–

ND, not determined.

all tumor volume detected with ultrasonography, computed tomography or both. As indicated in Figure 5, total volume of tumor tissue in the patients with AFP mRNA in blood (441 ± 103 cm^3) was significantly larger than those in patients without AFP mRNA in the circulation (87 ± 49 cm^3) ($p < 0.05$).

AFP concentration in serum was significantly increased in HCC patients with AFP mRNA in blood ($62{,}738 \pm 7{,}031$ ng/ml) compared with patients without AFP mRNA in peripheral blood (536 ± 240 ng/ml) (Fig. 6) ($p < 0.05$; Mann-Whitney U test).

Extrahepatic metastasis was detected by means of imaging techniques such as scintigraphy or computed tomography or on postmortem examination. Extrahepatic metastases were demonstrated in 6 patients among 17 cases with AFP mRNA but not in 16 patients without AFP mRNA in peripheral blood ($p < 0.05$; χ^2 test) (Fig. 6). When the patients were classified according to the presence or absence of extrahepatic metastases, AFP mRNA was detected in the blood of all six patients with extrahepatic metastasis, in contrast to 11 of 27 patients without extrahepatic metastases ($p < 0.05$; χ^2 test) (Table 4).

DISCUSSION

Because metastasis of carcinoma is one of the most important factors affecting prognosis, extensive trials to detect cancer metastasis have been carried out with tumor-associated genes or proteins. Hematogenous metastases of colon cancer and melanoma are demonstrated by staining of the tumor-specific epithelial cytokeratin protein (cytokeratin 18) in bone marrow (19) and the presence of transcription of tyrosinase gene in the circulation (20), respectively. In addition, tumor-specific genes such as bcr-abl hybrid for chronic myelogenous leukemia (7) and mutation of K-*ras* codon 12 for pancreatic cancer (8) are also used for detection of tumor cells in the circulation.

The presence of circulating tumor cells is known to be an indicator of hematogenous spread of tumor cells

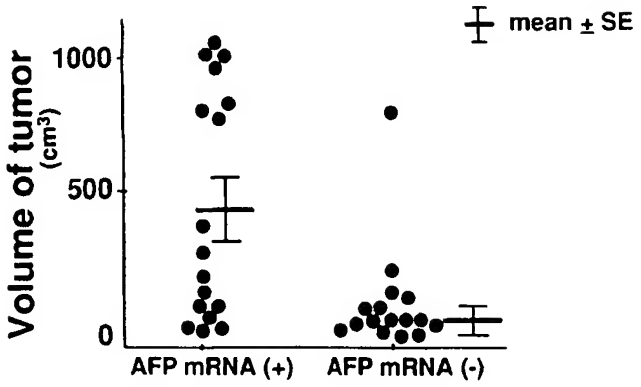


FIG. 5. Characteristics of patients with HCC in relation to total volume of tumor tissue. Total volume of tumor tissue in patients with AFP mRNA in blood was significantly larger than that in patients without AFP mRNA in blood ($p = 0.009$, Mann-Whitney U test).

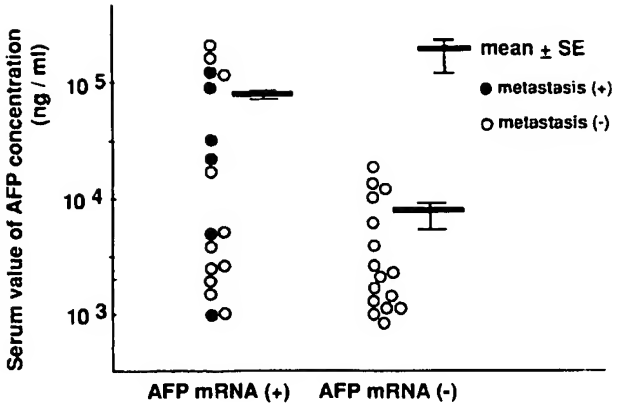


FIG. 6. Characteristics of patients with HCC in relation to serum AFP concentration. Serum AFP concentration was significantly increased in the patients with AFP mRNA in blood compared with patients without AFP mRNA in blood ($p = 0.040$, Mann-Whitney U test). Extrahepatic metastasis was detected in 6 of 17 patients with AFP mRNA but not in the patients without showing AFP mRNA in peripheral blood ($p < 0.05$, χ^2 test). Each S.E.M. value was calculated with the arithmetic mean instead of the geometric mean. Solid circles denote patients with extrahepatic metastasis; open circles denote patients without.

leading to metastasis at other organs. An enhanced serum AFP concentration is used for a screening of HCC (16) but does not reflect the presence of circulating tumor cells. Therefore an increased AFP concentration in serum cannot directly predict metastasis of HCC. In this study, we tried to demonstrate circulating HCC cells, which may be released from tumor foci into the circulation, by detecting the hepatocyte- or HCC-associated mRNA in the nuclear cell component of peripheral blood using nested RT-PCR because the number of circulating malignant cells is too low. In the preliminary experiments, we tried to demonstrate circulating HCC morphologically after collecting nuclear cells with a cytospinner and staining them by use of immunohistochemistry but failed.

Because albumin is known to be produced by hepatocytes (9), we tried to examine the presence of albumin mRNA in peripheral blood of normal healthy volunteers as a negative control. However, albumin mRNA was detected not only in liver tissue and HepG2 cells but also in the nuclear cell component of peripheral blood from normal volunteers on nested RT-PCR, suggesting that tiny amounts of albumin message are transcribed in nuclear blood cells such as lymphocytes and neutrophils. These results are consistent with the report of McLeod et al. (21), which indicated that albumin mRNA is detected not only in the liver but in several other organs—such as testis, uterus, placenta and yolk sac in rats—by RT-PCR. Therefore it is not rational to use albumin mRNA for detecting a small amount of circulating HCC with nested RT-PCR, and these results could prove wrong an earlier report stating that transcription of albumin mRNA is a marker of the presence of HCC cells (22). Although the albumin mRNA may be transcribed by neutrophils, lymphocytes or both, there is an another possibility: that there are circulating hepatocytes expressing albumin mRNA, but that the lack of AFP mRNA in peripheral blood of such individual may be due to the inability of normal hepatocytes to transcribe AFP mRNA.

We, then, sought AFP mRNA for detection of HCC in peripheral blood. AFP mRNA was detected in liver tissue and HepG2 cells but not in peripheral blood, even on nested RT-PCR. Because AFP mRNA was not demonstrated in the blood of normal healthy volunteers, AFP mRNA in peripheral blood may have indicated the presence of hepatocytes/HCC cells in the circulation. AFP mRNA was detected in the circulation of 52% of patients with HCC and in that of 15% and 12% of patients with cirrhosis and chronic hepatitis, respectively. The presence of AFP mRNA in a small number of patients with chronic hepatitis or cirrhosis may have been related to the fact that increased serum AFP concentrations are found in patients recovering from acute hepatitis or acute exacerbation of chronic hepatitis (23) and that a few injured or necrotic hepatocytes may have entered the circulation in these cases. To approach this possibility, we collected blood at different sites during cardiac catheterization. AFP mRNA in the blood of hepatic vein was demonstrated in 3 of 4 patients with liver diseases but in only 2 of 11 cases without liver diseases. However, AFP mRNA in the blood of inferior vena cava was detected in one case with cirrhosis only. These data suggest that injured or necrotic hepatocytes released into the circulation may be detected in blood taken at the hepatic vein. Because these cells might be removed from the circulation by macrophages in the lung, AFP mRNA was not any more detected at aorta or at inferior vena cava. However, many injured hepatocytes were released from the liver and spilled into the circulation after passage through the lung, AFP mRNA could be detected in peripheral blood.

We then examined the characteristics of the patients with HCC who showed AFP mRNA in peripheral blood.

TABLE 4. Characteristic of patients with HCC in relation to extrahepatic metastasis

Metastasis	AFP mRNA in peripheral blood			
	No.	Positive	Negative	% Positive
Present	6	6	0	100[a]
Absent	27	11	16	41

[a] $p < 0.05$ vs. those without metastasis (χ^2 test).

The incidence of AFP mRNA in blood was significantly increased in association with the size of tumors and serum AFP concentration. In addition, extrahepatic metastasis was demonstrated only among the patients showing AFP mRNA, not in the patients without AFP mRNA in peripheral blood. These results may have indicated that tumor cells were released from tumor foci into the circulation when tumor size increased. Furthermore, detection of AFP mRNA in blood may reflect the presence of HCC/hepatocytes in the circulation, which could lead to extrahepatic metastasis. On the other hand, serum AFP levels may not reflect the existence of extrahepatic metastasis but may be useful only in screening of HCC (16).

AFP mRNA was found in the peripheral blood of all cases (100%) of HCC with extrahepatic metastases and in 41% of the patients without extrahepatic metastases. These results could indicate that detection of AFP mRNA in peripheral blood is a useful marker to select the patients in whom extrahepatic metastases might develop, although further study of these patients will be necessary to determine whether extrahepatic metastases develop in the near future.

The efficacy of tumor metastasis is considered to depend on the balance between selected properties of tumor cells and reactivity of the host (24). In the process of metastasis, tumor cells are scattered from the original site, spreaded hematogenously, arrested at the small vessels and extravasated from the vessels. During these processes, tumor cells are attacked and destroyed by immunologically responsive cells (25) or by mechanical force. Thus, a moderate number of circulating tumor cells is needed to form foci of micrometastasis *in vivo* (26). Therefore the detection of cancer cells in blood does not always reflect the existence of metastatic foci. Moreover, metastatic foci at other organs may be too small to be detected, in some cases, with the imaging techniques used. Because the presence of micrometastases of HCC at other organs leads to poor prognosis in patients with HCC even after transplantation of the liver (6), prediction of micrometastasis on the basis of detection of circulating HCC (AFP mRNA in blood) before liver transplantation could be a useful tool for the selection of transplant recipients.

Acknowledgment: We thank the members of the cardiovascular section of our department for their aid in collecting blood during cardiac catheterization.

REFERENCES

1. Oka H, Kurioka N, Kim K, Kanno T, Kuroki T, Mizokuchi Y, Kobayashi K. Prospective study of early detection of hepatocellular carcinoma in patients with cirrhosis. HEPATOLOGY 1990;12: 680-687.
2. Tsuzuki T, Ogata Y, Iida S, Shimazu M. Hepatic resection in 125 patients. Arch Surg 1984;119:1025-1032.
3. O'Halpin D, Legge D, MacErlean DP. Therapeutic arterial embolization: report of five years' experience. Clin Radiol 1984; 35:85-93.
4. Shiina S, Tagawa K, Niwa Y, Unuma T, Komatsu Y, Yoshiura K, Hamada E, et al. Percutaneous ethanol injection therapy for hepatocellular carcinoma. Am J Roentogenol 1993;160:1023-1028.
5. Lee Y, Teer DA. Primary liver cancer: pattern of metastasis. J Surg Oncol 1987;36:26-31.
6. Ringe B, Wittekind C. The role of liver transplantation in hepatobiliary malignancy: a retrospective analysis of 95 patients with particular regard to tumor stage and recurrence. Ann Surg 1989;209:88-98.
7. Hirai H, Ishikawa F. Analysis of Ph1-positive leukemia by PCR [in Japanese]. Rinsho-Ketsueki 1991;32:319-326.
8. Tada M, Omata M, Kawai S, Saisho H, Ohto M, Sakai RK, Sninsky JJ. Detection of ras gene mutation in pancreatic juice and peripheral blood of patients with pancreatic adenocarcinoma. Cancer Res 1993;53:2472-2474.
9. Weiner FR, Czaja MJ, Giambrone MA, Wu CH, Wu GY, Zern MA. Development of molecular hybridization technology to evaluate albumin and procollagen mRNA content in baboons and man. HEPATOLOGY 1987;7:19S-25S.
10. Tamaoki T, Fausto N. Expression of the α-feto protein gene during development, regeneration and carcinogenesis. In: Stein G, Stein J, eds. Recombinant DNA and cell proliferation. New York: Academic Press, 1984:145-168.
11. Minghetti PP, Ruffner DE, Kuang WJ, Dennison OE, Hawkins JW, Beattie WG, Dugaiczyk A. Molecular structure of the human albumin gene is revealed by nucleotide sequence within q11-22 of chromosome 4. J Biol Chem 1986;261:6747-6757.
12. Sakai M, Morinaga T, Urano Y. The human α-fetoprotein gene: sequence organization and the 5'flanking region. J Biol Chem 1985;260:5055-5060.
13. Tsutsumi T, Nakao K, Mitsuoka S, Hamasaki K, Tsuruta S, Shima M, Nakata K. Regulation of albumin and α-feto protein gene expression by colloid osmotic pressure in human hepatoma cells. Gastroenterology 1993;104:256-262.
14. Camper SA, Godbout R, Tilghman SM. The developmental regulation of albumin and α-fetoprotein gene expression. Prog Nucleic Acid Res Mol Biol 1989;36:131-143.
15. Peng SY, Lai OL, Chu JS, Lee PH, Tsung PT, Chen DS, Hsu HC. Expression and hypomethylation of α-fetoprotein gene in unicentric and multicentric human hepatocellular carcinoma. HEPATOLOGY 1993;17:35-41.
16. Okuda K, Kotoda K, Obata H, Hayashi N, Hisamitsu T. Clinical observations during a relatively early stage of hepatocellular carcinoma, with special reference to serum α-fetoprotein levels. Gastroenterology 1975;69:226-234.
17. Pinthanond D, Petchclai B. A preserved leukocyte preparation for quality control. Am J Clin Pathol 1975;63:32-34.
18. Lawn RM, Efstratiadis A, O'Connel C, Maniatis T. The nucleotide sequence of the human β-globin gene. Cell 1980;21:647-651.

19. Lindemann F, Schlimok G, Dirschedl P, Witte J, Riethmuller G. Prognostic significance of micrometastatic tumor cells in bone marrow of colorectal cancer patients. Lancet 1992;340:685-688.
20. Smith B, Selby P, Southgate J, Pittman K, Bradley C, Blair GE. Detection of melanoma cells in peripheral blood by means of reverse transcriptase and polymerase chain reaction. Lancet 1991;338:1227-1229.
21. McLeod JF, Cooke NE. The vitamin D–binding protein, α-feto protein, albumin multigene family: detection of transcripts in multiple tissues. J Biol Chem 1989;264:21760-21769.
22. Carr BI, Kar S. An assay for hepatoma micrometastasis: albumin gene expression in peripheral blood from patients with advanced hepatocellular carcinoma (HCC) [Abstract]. HEPATOLOGY 1991;14: 108A.
23. Bloomer JR, Waldmann TA, McIntire KR, Klatskin G. α-fetoprotein in nonneoplastic hepatic disorders. JAMA 1975;233: 38-41.
24. Paul BU, Lee CL. Organ preference of metastasis. The role of organ-specifically modulated endothelial cells. Lab Invest 1988; 58:379-387.
25. Shiratori Y, Nakata R, Okano K, Komatsu Y, Shiina S, Kawase T, Sugimoto T, et al. Inhibition of hepatic metastasis of colon carcinoma by asialo GM_1–positive cells in the liver. HEPATOLOGY 1992;16:469-478.
26. Tominaga T, Yoshida Y, Kitamura M, Kosaki G. Liver metastasis of colon 26 cells implanted into the superior mesenteric vein in mice. Jpn J Cancer Res 1987;78:846-850.

A12

Vol. 207, No. 2, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 15, 1995 Pages 813-818

SENSITIVE ASSAY FOR DETECTION OF HEPATOCELLULAR CARCINOMA ASSOCIATED GENE TRANSCRIPTION (ALPHA-FETOPROTEIN mRNA) IN BLOOD

Masayuki Matsumura [1]*, Yasuro Niwa [1], Yohko Hikiba [2], Ken-ichi Okano [2], Naoya Kato [1], Shuichiro Shiina [1], Yasushi Shiratori [1] and Masao Omata [1]

[1] Second Department of Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan 113,

[2] Div. of Gastroenterology and Hepatology, Institute of Adult Disease, Asahi Life Foundation, Nishi-Shin'juku, Shin'juku, Tokyo, Japan

Received January 6, 1995

SUMMARY: The sensitivity of RT-PCR and nested RT-PCR for detection of circulating hepatocellular carcinoma was assessed by demonstrating the tumor cell-associated gene transcription, alpha-fetoprotein mRNA, in the nuclear cells of peripheral blood. When HepG2 cells were mixed in blood, 100-1000 tumor cells/5ml of blood could be detected by RT-PCR, in contrast to 1-10 tumor cells/5ml of blood by nested RT-PCR. In addition, $2x10^4$ copies of AFP mRNA were found in one HepG2 cell when analyzed by the quantitative nested RT-PCR assay. Thus, the nested RT-PCR assay could provide a useful tool for detecting a tiny amount of circulating tumor cells in patients with hepatocellular carcinoma presenting extra-hepatic metastasis.

Prognosis of patients with hepatocellular carcinoma (HCC) is estimated by several factors, such as histological differentiation of tumor cells, extent of tumor size, and extent of lymphatic or hematogenous spread. Venous invasion of tumor cells, first step to induce blood-born metastasis, is one of the important factor affecting prognosis of these patients. Although the efficacy of tumor metastasis is considered to depend on the balance between selected properties of tumor cells and reactivity of the host against tumor cells. In the process of metastasis, tumor cells are scattered from the original site, spreaded hematogenously and arrested at small vessels. Thus, detection of tumor cells in the circulation might predict metastasis of tumor. However, the number of

*Correspondence. Fax:03-3814-0021.

0006-291X/95 $5.00

tumor cells in circulation could be extremely too small to be detected morphologically.

Recently, tumor associated gene in the circulating tumor cells (reflecting the presence of tumor cells in the circulation) could be detected by PCR in patients with leukemia (1), or in patients with pancreatic cancer with distant metastasis (2). Thus, detection of hepatocellular carcinoma associated gene in the circulation might be related to the hematogenous spreading metastasis of HCC, even though overt metastasis could be obscure.

In the present study, the difference of sensitivity among RT-PCR and nested RT-PCR assay for detection of tumor cells in blood has been estimated.

MATERIALS AND METHODS

Cell line; HepG2 cell line (3) (a gift from Dr. Makoto Noda in Riken Cell Bank; RCB 459) was maintained in Dulbecco's Minimum Essential Medium (Gibco-BRL, Grand Island, NY) containing 10% fetal bovine serum (Gibco-BRL).

Peripheral blood from healthy volunteer was collected in a disposable syringe with 0.1% EDTA. One thousand HepG2 cells were added into 5ml of blood, and serial dilution of tumor cells was made. Each dilution sample contained 1,000, 100, 10 and 1 HepG2 cells per 5ml of blood.

Preparation of nuclear cells from peripheral blood; After adding one ml of 5% dextran-saline solution into 5 ml of blood (4), the syringe was stood still for 30 min at room temperature. Supernatant was collected and centrifugated at 500 x g for 25 min. Residual erythrocytes was lysed by adding distilled water and isotonicity was then restored after 25 sec by adding the same volume of 1.8% NaCl solution. After centrifugation at 350 x g for 5 min, the cells were immediately frozen using liquid nitrogen and stored at -80°C until use.

Extraction of RNA and synthesis of complementary DNA (cDNA); By using RNAsol B (BIOTECK LABOLATORIES, Houston, TX), total RNA was extracted from nuclear cell component of peripheral blood. About 5 μg of RNA was extracted from 5 ml of blood. In addition, one μg of RNA was extracted from $5x10^4$ HepG2 cells.

One μg of RNA, which was heated at 95°C for 5 min and rapidly cooled on ice, was added in a buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.25 μg random primer (hexamer) (Takara Biochemicals, Kyoto, Japan), 18 units RNAse inhibitor (Takara Biochemicals), and 200 units M-MLV reverse transcriptase (Gibco-BRL). The volume was adjusted to 10 μl by adding DEPC treated water. Complementary DNA (cDNA) was synthesized by incubating the mixture at 42 °C for 60 min, and it was subsequently heated at 95°C for 5 min to inactivate reverse transcriptase. Complementary DNA was kept at -20°C until use.

Sensitivity of RT-PCR and nested RT-PCR

Polymerase chain reaction (PCR); Primers of AFP (5) mRNA used in RT-PCR were 5'- CTCTTCCAGCAAAGCACACTTC -3' (#7: upper stream) and 5'-CTCTTCAGCAAAGCAGACTTC -3' (#8: down stream). Integrity of RNA

was checked by amplifying ß-globin (6) mRNA with primers 5'-ACCCAGAGGTTCTTTGAGTC -3' (#26: upper stream) and 5'-TCTGATAGGCAGCCTGCACT - 3' (# 27: down stream).

Ten μl of cDNA solution was mixed with 40 μl of PCR reaction mixture containing 50 mM Tris-HCl (pH 8.3), 44 mM KCl, 1.1 mM $MgCl_2$, 0.013% gelatin, 12.5 pmol each primer (#7 and #8, or #26 and #27) and 1 unit Taq DNA polymerase (Takara Biochemicals). After initial denaturation at 95°C for 5 min, PCR was performed according to the temperature profile (93°C for 30 sec, 54°C for 45 sec and 72°C for 45 sec) for 35 cycles. The reaction was terminated by heating at 72°C for 7 min and cooled to 4°C.

Nested RT-PCR; Primers of AFP mRNA used in the nested PCR were 5'-GCTGACATTATTATCGGACAC-3' (#M1: upper stream) and 5'-AGCCTCAAGTTGTTCCTCTGT-3' (# M2: down stream).

Five μl of the amplified product of the RT-PCR was mixed with 5 ul of second PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 10 mM $MgCl_2$, 100 μg/ml gelatin), 12.5 pmol of each primer (# M1 and # M2) ,1 unit of Taq DNA polymerase and diluted to 50μl with distilled water. Themperature profile of nested PCR was the same one as described above.

Gel electrophoresis; Amplified product was electrophoresed on 3% agarose gels and stained with ethidium bromide. Size of the amplified product of AFP mRNA was 359 bp (PCR), 282 bp (nested PCR), and 283 bp for ß-globin.

Quantification of AFP mRNA in HepG2 cell

Synthesis of internal standard; RNA was extracted from HepG2 cells and was applied to RT-PCR. New restriction site (cut by Hind III) was introduced in the middle of amplified product according to the method described by Horton and Pease (7). The product was then inserted into pBluescript-II vector under the promoter of T7 RNA polymerase, and its sequence was confirmed by the ALF DNA Sequencer (Pharmacia, Uppsala, Sweden). After linearizing the plasmid, RNA was synthesized using T7 RNA polymerase. The RNA was quantified and diluted serially with DEPC-treated water. It was used as internal standard of quantitative PCR assay.

After adding the serially-diluted (10^4 to 10^{10} copies) internal standards to each of 1 μg RNA extracted from HepG2 cells, the mixture was reverse transcribed followed by nested PCR as described above. Amplified product was then cut with Hind III and was electrophoresed on 3% agarose gel. Three bands of 282 bp (wild type), 170 and 112 bp (both are derived from the internal standard) were demonstrated. An amount of AFR mRNA was calculated by comparison the intensity of the band of wild type with those of internal control (summation of two bands) (Figure 3).

RESULTS

Absence of AFP mRNA in blood of healthy volunteers; AFP mRNA was not demonstrated in nuclear cell component of peripheral blood of healthy volunteers both by RT-PCR and even by nested RT-PCR (Fig. 1).

Detection of AFP mRNA in diluted samples of HepG2 cells; As shown in Fig.

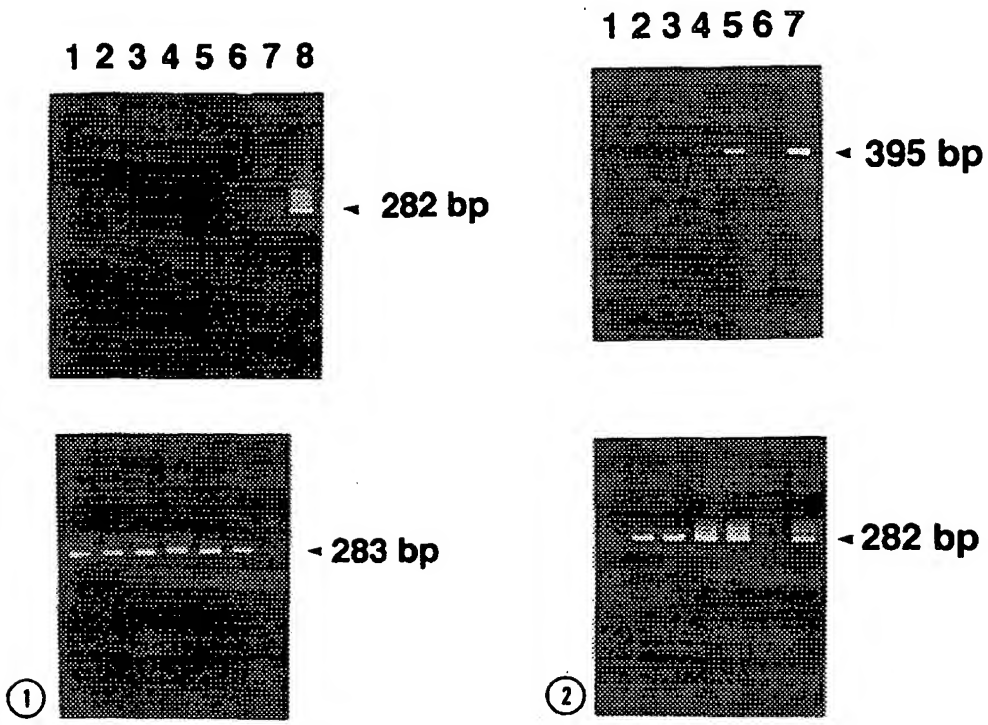


Fig.1. Amplification of AFP mRNA in blood of healthy volunteers.
Upper panel shows the result of nested PCR with primers #M1 and #M2 (AFP). Amplified product of PCR is 282 bp.
Lower panel shows the result of PCR with primers #26 and #27 (ß-globin). Amplified product is 283 bp.
Lanes 1-6: blood from healthy volunteers, lane 7: negative control (without RNA), lane 8: positive control (RNA from HepG2 cells).

Fig.2. Sensitivity of RT-PCR and nested RT-PCR for detection of HepG2 cells in blood.
Lanes 1-5: Dilution of HepG2 cells. Each lane includes (from left to right) 0, 1, 10, 100 and 1,000 HepG2 cells in 5 ml of normal blood, respectively, lane 6: negative control (without RNA), lane 7: positive control (1 μg of RNA from HepG2 cells).

2, AFP mRNA was detected in 5 ml of blood containing 100 tumor cells by the RT-PCR. In contrast, AFP mRNA was demonstrated in 5 ml of blood containing 1-10 tumor cells only by the nested RT-PCR. The sensitivity of nested RT-PCR was enhanced by around 10-100 folds, as compared with RT-PCR only.

Absolute number of AFP mRNA; As indicated in Fig. 3, 10^9 copies of AFP mRNA were found in 1 μg RNA of HepG2 cells. As one μg RNA was extracted from $5x10^4$ HepG2 cells, around $4x10^3$ copies of AFP mRNA were found in one HepG2 cell.

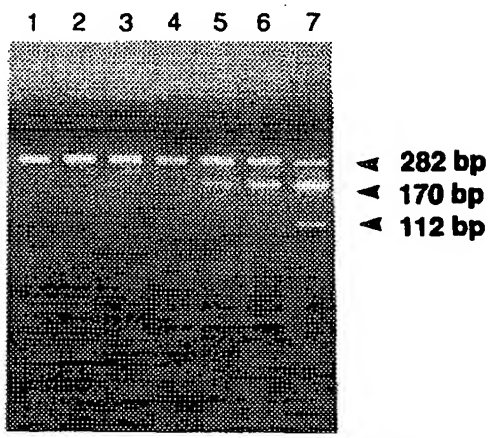


Fig.3. Quantification of AFP mRNA in HepG2 cell.
Lanes 1-7: Each lane includes the serially diluted-internal standard; (from left to right) 10^4, 10^5, 10^6, 10^7, 10^8, 10^9 and 10^{10} copies per sample.

DISCUSSION

In order to detect tumor cells in blood, demonstration of tumor associated gene has been developed using molecular techniques (1,2). In the recent study, albumin mRNA in blood has been found as a marker of circulating hepatocytes (8). There are some papers indicating that the production of AFP is increased in hepatocellular carcinoma cell lines such as HepG2 (3), and expression of AFP gene is reported to be increased during carcinogenesis (9). Thus, we have developed PCR assay for detection of HCC-associated tumor gene transcript, AFP mRNA, in nuclear cell component of blood, since the number of circulating tumor cells in patients with HCC demonstrating metastasis at distant organs might be extremely too small. In order to elucidate the sensitivity of RT-PCR and nested RT-PCR for detection of HCC cells in blood, HepG2 cells were mixed with blood, as HepG2 cells were established from hepatoblastoma and produces adequate amount of AFP (3). AFP mRNA could be detected by RT-PCR when 100 tumor cells are present in 5 ml of blood, in contrast to 1-10 tumor cell per 5 ml of blood by nested RT-PCR, suggesting that the sensitivity of nested RT-PCR is around 100 folds enhanced as compared with RT-PCR. Thus, if HepG2 cells were circulating in a body (total volume of blood; around 5,000 ml), their presence could be detected by RT-PCR when more than 10^5 tumor cells present in circulation. On the other hand, AFP mRNA could be detected by nested RT-PCR even when only 10^3 tumor cells are present in the circulation. Although a moderate number of circulating tumor cells might be present as assessed from the calculation of detection of HepG2 cells, there might be several steps for establishment of metastasis (10) such as adhesion of tumor cells at

vascular endothelium, migration into the extracellular space, and proliferation at the metastatic foci. During these process, immunological reaction of host organs takes place, and some tumor cells might be damaged by immunocompetent cells or by mechanical force of blood flow or by platelet aggregation.

Furthermore, the quantitative analysis has been performed by competitive RT-PCR (11) to estimate an amount of AFP mRNA in HepG2 cell, indicating that around $2x10^4$ copies of AFP mRNA were included in one HepG2 cell. As one HepG2 cells per 5 ml of blood could be detected by this nested RT-PCR, around 4,000 copies of AFP mRNA in one ml of blood could be demonstrated by this assay.

These results suggest that high sensitivity detection assay of nested RT-PCR could be useful for demonstration of hematogenous spreading tumor cells. In addition, further prospective study is needed for prediction of metastasis in patients with HCC by detecting circulating tumor cells.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Health and Welfare; the Ministry of Education, Culture and Science, Japan.

REFERENCES

1. Hirai, H., Ishikawa, F. (1991) Rinsho-Ketsueki (in Japanese) 32, 319-326.
2. Tada, M., Omata, M. (1993) Cancer Res 53, 2472-2474.
3. Aden, D.P., Fogel, A. (1979) Nature 282, 615-616.
4. Pintthanond, D. and Petchclai, B. (1975) American Journal of Clinical Pathology 63, 32- 34.
5. Sakai, M., Morinaga, T. (1985) J Biol Chem 260, 5055-5060.
6. Lawn, R. M., Efstratiadis, A. (1980) Cell 21 , 647-651.
7. Horton, R.M., Pease, L.R. (1991) In Directed Mutagenesis. (McPherson, M.J. Ed.) pp.217- 247. Oxford University Press, Oxford.
8. Hillaire, S., Barbu, V. (1994) Gastroenterology 106, 239-242.
9. Tamaoki, T, Fausto, N. (1984) Recombinant DNA and cell proliferatiion. (Stein, G. Stein, J. Ed.) pp 145-168. Academic Press, New York.
10. Hart, I.R., Saini, A. (1992) Lancet 339, 1453-1457.
11. Becker-Andre, M., Hahlbrock, K. (1989) Nucleic Acids Res 22, 9437-9446.

A13

Ann. Rech. Vét., 1978, **9** *(4)*, 825-830.

TUMOR-ASSOCIATED ANTIGEN AND CELL SURFACE MARKER IN CELLS OF BOVINE LYMPHOSARCOMA

M. ONUMA *, I. TAKASHIMA **and C. OLSON **

* *Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060, Japan*
** *Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin 53706 U.S.A.*

Résumé

ANTIGENE ASSOCIE AUX TUMEURS ET MARQUEUR DE LA SURFACE CELLULAIRE DANS LES CELLULES DE LYMPHOSARCOME BOVIN. — La détection d'antigène associé aux tumeurs et de marqueurs de surface des cellules B a été tentée dans des cellules lymphoïdes du sang périphérique (PBL) et des ganglions lymphatiques provenant de bovins infectés par le virus de la leucose bovine (BLV), et chez des bovins cliniquement normaux.

Un antigène spécifique associé à la tumeur a été trouvé dans les cellules PBL et les cellules de tumeurs lymphoïdes des bovins ayant la forme adulte du lymphosarcome (ALS), au moyen d'un sérum anti ALS. L'antigène a aussi été détecté dans les cellules PBL de bovins infectés par le BLV. Une réaction faible à l'antigène a été observée dans deux cas sur cinq de tumeur du veau. Aucune réaction significative pour l'antigène associé à la tumeur n'a été observée de la part des cellules lymphoïdes néoplasiques d'ALS dans les formes thymiques et cutanées. Le pourcentage de cellules B dans les cellules PBL et les ganglions lymphatiques d'ALS était plus élevé que chez les bovins normaux. Cependant, il semble qu'il y ait dans certains cas une perte des marqueurs de surface des cellules B dans certains cas d'ALS. On a observé une bonne relation entre l'augmentation du pourcentage de cellules B et l'apparition de l'antigène associé aux tumeurs dans les cellules PBL des bovins infectés par le BLV. Dans les cas sporadiques, le pourcentage de cellules B était plus faible parmi les cellules lymphoïdes de PBL et des ganglions que chez des bovins normaux.

Introduction.

Bovine lymphosarcoma is a neoplasm of lymphoid tissue associated with BLV. Experimental inoculation with BLV can induce lymphocytosis or persistent lymphocytosis in young calves (Miller *et al.*, 1972) and lymphosarcoma in sheep (Olson and Baumgartner, 1976). The specific tumor-associated antigen was found on neoplastic lymphoid cells from cattle and sheep with lymphosarcoma (Onuma and Olson, 1977 b). The antigen was also found in peripheral blood lymphocytes (PBL) of BLV infected cattle showing no evidence of lymphosarcoma. An increased B cell number was recognized in PBL and lymph node cells of cattle with persistent lymphocytosis and cattle with the adult form of lymphosarcoma

(ALS) cases (Muscoplat *et al.*, 1974 ; Takashima *et al.*, 1977 ; Weiland and Straub, 1975). Loss of normal B cell surface marker was also suggested in lymphoid tumors from some ALS cases (Takashima *et al.*, 1977). The purpose of the present report is to describe the relation between the appearance of tumor-associated antigen and the alteration in B cell number in neoplastic lymphoid cells.

Materials and Methods.

Animals.

Tissues from cattle with ALS (3163, 3167, 3169, 3170, 3183, 3191 and 3192) were obtained from an abattoir (Oscar Mayer and Co. Madison Wis., U.S.A.) ; and those with the calf form of lymphosarcoma (CLS : 3178 and 3182) were from farmers in Wisconsin, U.S.A. Tissue from cattle with the thymic form of lymphosarcoma (TLS ; 3185) were provided by Dr. W.J. Jarrett, University of Glasgow, Glasgow, U.K. Tissues from cattle with 3 ALS (117, 128 and 129), one TLS (126) and two cases of the skin form of lymphosarcoma (SLS ; 114 and 127) were obtained from farmers in Hokkaido, Japan. Lymphosarcoma was diagnosed by both gross and histological examinations. PBL of BLV-infected cattle, either experimentally infected (289, 294, 340 and 500) or naturally infected (2197) were collected from an experimental herd at the Universihy of Wisconsin, Madison, Wis., U.S.A. PBL from a naturallk BLV-infected cow (131) came from an experimental herd at the Kitasato University, Towada, Japan. PBL from naturally BLV-infected cattle (I-7, I-11, I-23 and ch-4) were collected from farmers in Hokkaido, Japan. Tissues from 4 clinically normal cattle with histologically normal lymph nodes and PBL were also collected from abattoirs. Cattle with lymphosarcoma 3169, 3178, 3182 and 3185 were described in the previous paper (Onuma and Olson, 1977a).

Table 1. — Tests for tumor-associated antigen and sIg bearing cells in neoplastic lymphoid cells of ALS.

Case No.	Form	BLV antibodies	Tissues	BLV	B cell[a] (%)	Tumor-associated antigen[b] fixed-cell	antigen[b] living cell	Viability (%)
3163	ALS	+	lymph node		97	+ +	+	93
3167	"	+	PBL	+	27	+ +	+ +	99
			lymph node		71	+	+ +	79
3169	"	+	PBL	+	10	+	+ +	90
			lymph node	+	65	+	+	85
3170	"	+	PBL	—	16	+	+	88
			lymph node	—	87	±	+ +	92
3183	"	+	PBL	+	2	+ +		93
3191	"	+	PBL		75	+		89
			lymph node		52	+		92
3192	"	+	PBL		84	+ +		93
			lymph node		84			96
103	"	+	PBL		75			95
			lymph node	+	91			98
			PBL	+	58			98
107	"	+	lymph node	+	15	+		95
			abomasum		18			95
			PBL	+	72			96
119	"	+	lymph node	+	20	+ +		89
			heart	+	56			92

a) Percentage of surface immunoglobulin-positive cells.
b) Antigen was detected by fluorescent antibody test using anti-ALS serum. Results were expressed as ± to + + : ±, questionable fluorescence ; + + bright green fluorescence ; —, none.

Table 2. — Tests for tumor-associated antigen and sIg bearing cells in neoplastic lymphoid cells of CLS, TLS and SLS.

Case No.	Form	BLV antibodies	Tissues	BLV	B cell (%)	Tumor-associated antigenc Fixed cell	Tumor-associated antigenc Living cell	Viability (%)
3178	CLS	−	PBL	+	2.0	+	+	90
3182	"	−	PBL	−	3.0	+		96
117	"	−	lymph node	−	0.1			70
128	"	−	PBL	−	2.4	−	−	75
			lymph node	−	1.0	−	±	90
129	"	−	PBL	−	2.0	−	−	80
			lymph node	−	0.7	−	±	93
3185	TLS	−	PBL	−	0.6	±	+	90
126	"	−	lymph node	−	5.5	−		85
114	SLS	−	PBL	−	3.2	−		98
			lymph node	−	1.0	−		90
127	"	−	PBL	−	7.5	−	−	86
			skin tumor	−	0.2	−	−	100

Results are expressed as in Table 1.

Serological tests.

To detect antibodies to BLV, immunodiffusion test was done using glycoprotein antigen as previously described (Onuma *et al.*, 1977). BLV was detected in short-term lymphocyte culture or long-term monolayer culture by fluorescent antibody (FA) or complement fixation (CF) tests (Miller and Van der Maaten, 1974 ; Onuma *et al.*, 1977). Recently, the syncytium assay has been used to detect BLV (Diglio and Ferrer, 1976).

Cell preparations.

Fresh bovine tumor was collected in Eagle's minimal essential medium with 10 % fetal calf serum and antibiotics. Tumor cells were teased out and filtered through gauze to obtain suspensions of single cells as described previously (Onuma and Olson, 1977b). Lymphocytes were separated from blood by a Ficoll-Hypaque gradient or Ficoll-Conray gradient as described by Boyüm (1968). Viability of these cell preparations was determined by trypan blue exclusion test. Cell preparations with viability of more than 70 % from lymph nodes and more than 86 % from PBL were studied for detection of surface immunoglobulin (sIg) bearing cells.

Surface immunoglobulin (sIg) immunofluorescence.

Lymphocytes (2×10^6/ml) in PBS were stained with fluorescein-conjugated anti-bovine IgG serum at 37°C for 30 minutes. The number of positive cells was determined by counting 200 cells with the fluorescence microscope under oil immersion.

Detection of tumor-associated antigen.

The FA test was performed to detect the tumor-associated antigen in both living cells and fixed cells using anti-ALS serum (Onuma and Olson, 1977 b). Acetone-fixed cells were incubated with anti-ALS serum at 37°C for 30 minutes, washed and then stained with FITC-conjugated anti-bovine γ-globulin rabbit serum. A single cell suspensiGn from fresh tumor and PBL was prepared as described above. Living cells were stained with FITC conjugated anti-ALS γ-globulin.

Results

Ten ALS cases were examined by FA test

Table 3. — Tests for tumor-associated antigen and sIg bearing cells in lymphoid cells of BLV-infected and normal cattle.

Case No.	Form	BLV antibodies	Tissues	BLV	B cell (%)	Tumor-associated antigen Fixed cell	Tumor-associated antigen Living cell	Viability (%)
289	Exp. Infect.	+	PBL	+	55	+		92
294	"	+	"	+	28	−	−	97
340	"	+	"	+	30	−	−	99
500	"	+	"	+	58	+		94
2197[a]	Natural Infect.	+	"	+	59	+ +	+ +	92
131[a]	"	+	"	+	70		+	97
I-7	"	+	"		45		−	95
I-11	"	+	"		26		−	87
I-23	"	+	"	+	75		+	88
ch-4	"	+	"	+	93		+	98
3187	Normal	−	PBL	−	21	−		89
			Lymph node		24			84
3189	"	−	Lymph node	−	22	−	−	75
3190	"	−	Lymph node	−	17	−	−	84
H-1	"	−	PBL	−	30	−	−	82

Results are expressed as in Table 1. a) Persistent lymphocytosis was found.

using ALS antiserum (Table 1). Tumor-associated antigen was found on the membrane and in the cytoplasm of lymph node cells and PBL in all ALS cases tested. Antibody to BLV glycoprotein antigens was detected in sera from all of the ALS cases, and BLV antigen was detected in lymph nodes or PBL from 6 of 7 cases. Tumor cells with viability of more than 79 % of cells were studied for detection of sIg bearing cells. As shown in Table 1, increased B cell percentage was found in 9 of 10 cases. However, different B cell percentages were observed in different preparations from the same animals. In case No. 3183, the percentage of B cells was lower than that in normal cattle.

Nine sporadic bovine leukosis cases including 5 CLS, 2 TLS and 2 SLS were examined by FA test using ALS antiserum for detection of the tumor-associated antigen (Table 2). Weak or questionable positive reaction was observed in 4 CLS and one TLS cases but not SLS cases. None of these sporadic cases had antibody to BLV antigen. The sIg bearing cells of these cases were also examined. The percentages of sIg bearing cells from these cases were lower than those from the normal bovine cells. PBL from 10 BLV infected cattle showing no evidence of tumor were examined for the tumor-associated antigen using ALS antiserum (Table 3). Six cases were positive for the antigen. The B cell percentage of these positive 6 cases was higher than that of control BLV negative animals. For the other 4 cases, B cell percentage was in normal range except No. I-7. Cases 2197 and 131 showing persistent lymphocytosis and a high percentage of B cells gave positive reaction for the tumor-associated antigen. PBL from 10 more BLV infected cattle showing no evidence of tumor were also examined. B cell percentage was in normal range and no positive reactions were found (data was not shown).

Discussion

Specific tumor-associated antigen in lymphoid cells of BLV-infected cattle were described in a previous paper (Onuma and Olson, 1977 b). In ALS cases, the tumor-associated antigen was found on the membrane and in the cytoplasm of neoplastic lymphoid cells. An increased B cell number was recognized in ALS cases, however, different B cell percentages were observed in diffe-

rent preparations from the same animal in the present observation. When B cells were detected by both erythrocyte antibody-complement (EAC) rosette, and sIg immunofluorescence assays, EAC rosettes positive cells were fewer than sIg positive cells in the same preparation of ALS cases (Takashima *et al.*, 1977). These neoplastic cells with the tumor-associated antigen are B cell origin and may have lost their normal B cell surface marker during neoplastic transformation.

With regard to sporadic bovine leukosis, the etiology of the disease is still unknown. A weak reaction for the tumor-associated antigen was found in 2 of 5 cases of CLS. No significant reaction for the antigen of lymphosarcoma cells from ALS was found in the thymic and skin forms. The evidence that the neoplastic lymphoid cells from the sporadic leukosis could not be identified as B cells suggests that the neoplastic cell from these cases may not be of B cell origin. The tumor-associated antigen was also found in PBL from BLV-infected cattle showing no evidence of lymphosarcoma. B cell percentage in PBL from these cases in which the antigen was found was higher than that of BLV negative animals. However, the antigen was not detected in PBL from cattle showing no significant increase in B cell percentage. Therefore, a good correlation can be pointed out between an increase in B cell percentage and the appearance of the antigen. Case 289 which was experimentally BLV-infected, had the antigen but no lymphosarcoma when tested. However, three years later and nearly 11 years after it was inoculated it died with lymphosarcoma. The demonstration of this kind of antigen on PBL from BLV-infected cattle with no evidence of tumor may help the development of a new diagnostic test for bovine leukosis.

Acknowledgements.

We wish to express our appreciation to Drs. S. Ichijo, Department of Veterinary Internal Medicine, Obihiro UnivFrsity, Agriculture and Veterinary Medicine, Obihiro, Japan, M. Sonoda, College of Dairy Agriculture, Ebetsu, Japan and T. Yoshikawa, Kitasato University, Towada, Japan in obtaining materials. We also greatly appreciate the technical assistance of Sally Bertelson and Yuko Matsuzawa. This work was supported in part by the College of Agriculture and Life Sciences, University of Wisconsin, in part by USPHS Research Grant CA 13628 from the National Cancer Institute, and in part by Grant 257133 from the Ministry of Education, Science and Culture, Japan.

Summary

The detection of tumor-associated antigen and B cell surface marker was attempted in lymphoid cells of peripheral blood (PBL) and lymph nodes obtained from cattle with lymphosarcoma, cattle infected with bovine leukosis virus (BLV), and clinically normal cattle.
As a result, specific tumor-associated antigen was found in PBL and lymphoid tumor cells from cattle with the adult form of lymphosarcoma (ALS) using anti-ALS serum. The antigen was also detected in PBL of BLV-infected cattle. A weak reaction for the antigen was found in 2 of 5 cases of calf form. No significant reaction for the tumor-associated antigen of neoplastic lymphoid cells from ALS was found in the thymic and skin forms. The percentage of B cells in PBL and lymph nodes from ALS was higher than that in normal cattle. However, a loss of B cell surface marker was suggested in some ALS cases. A good relation was obtained between an increased B cell percentage and an appearance of the tumor-associated antigen in PBL of BLV-infected cattle. In lymphoid cells of PBL and lymph nodes from sporadic cases, the percentage of B cells was lower than that observed in normal cattle.

References

BOYÜM A., 1968. Separation of leukocytes from blood and bone marrpw. *Scand. J. Clin. Lab. Invest.*, **21**, (suppl. 97), 77-88

DIGLIO C.A., FERRER J.F., 1976. Induction of syncytia by the bovine C-type leukemia virus. *Cancer Res.*, **36**, 1056-1067.

MILLER L.D., MILLER J.M., OLSON C., 1972. Inoculation of calves with particles resembling C-type virus from cultures of bovine lymphosarcoma. *J. Natl. Cancer Inst.*, **48**, 423-428.

MILLER J.M., VAN DER MAATEN M.J., 1974. A complement fixation test for the bovine leukemia (C-type) virus. *J. Natl. Cancer Inst.*, **53**, 1699-1702.

MUSCOPLAT C.C. *ET AL.*, 1974. Lymphocyte surface immunoglobulin : Frequency in normal and lymphocytotic cattle. *Am. J. Vet. Res*, **35**, 593-595.

OLSON C., BAUMGARTENER L.E., 1976. Pathology of lymphosarcoma in sheep induced with bovine leukemia virus. *Cancer Res.*, **36**, 2365-2373.

ONUMA M. *ET AL.*, 1977. Fetal infection with bovine leukemia virus in sheep. *Cancer Res.*, **37**, 4075-4081.

ONUMA M., OLSON C., 1977 a. Bovine leukemia virus antigen in bovine lymphosarcoma cell cultures. *In :* « Bovine leukosis : various methods of molecular virology » (A. Burny, ed.) pp. 95-117. *Commission of the European Communities, Luxembourg.*

ONUMA M., OLSON C., 1977 b. Tumor-associated antigen in bovine and ovine lymphosarcoma. *Cancer Res.*, **37**, 3249-3256.

TAKASHIMA I. *ET AL.*, 1977. B-lymphocytes and T-lymphocytes in three types of bovine lymphosarcoma. *J. Natl. Cancer Inst.*, **59**, 1205-1209.

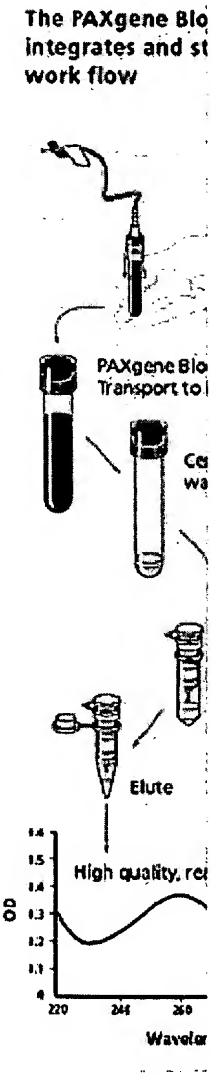
WEILAND F., STRAUB O.C., 1975. Frequency of surface immunoglobulin bearing lymphocytes in cattle affected with bovine leukosis. *Res. Vet. Sci.*, **19**, 100-102.

A14

PreAnalytiX
A QIAGEN / BD Company

PAXgene™ Blood RNA System

The PAXgene Blood RNA System is a revolutionary, enabling technology that consolidates and integrates the key steps of whole blood collection, nucleic acid stabilization, and RNA purification. By minimizing the unpredictability associated with RNA processing, the system provides enhanced accuracy of intracellular RNA analysis.



Blood Collection

The PAXgene system is standardized on convenient BD Vacutainer™ technology, enhancing patient and health care worker safety, providing sample protection, and facilitating consistent blood volumes.

Stabilization

The PAXgene tube contains a proprietary reagent that immediately **stabilizes intracellular RNA for 3 days at room temperature (18-25°C) and 5 days at 2-8°C.** The ability to eliminate freezing, batch samples, and minimize processing urgency greatly enhances lab efficiency.

Purification

The PAXgene kit uses a unique version of QIAGEN's proven separation and purification technology. **The purification process is designed to provide high quality RNA from samples that have been stabilized in the PreAnalytiX reagent.**

Standardization

The PAXgene Blood RNA System provides pharmaceutical, CRO, and research institutions with a completely integrated approach to RNA purification. No special kits. No uncertainty. One integrated product – from blood collection to pure RNA.

Instructions for Use

"PAXgene 96 Blood RNA and PAXgene Blood RNA MDX kits are intended for general laboratory use. PA RNA, and PAXgene DNA Systems are intended for Research Use Only. The product's performance char and efficacy have not been established and have not been submitted for review by the FDA. Therefore be used for patient diagnosis or evaluation of disease."

A15

"Section 6.1.2 Ribonuclease degradation", excerpt from *Essential molecular biology.* By Terry Brown. Published by Oxford University Press, 1999.

6.1.2 Ribonuclease degradation

Protocol 5 typically provides intact RNA suitable for Northern blots (14). The dextran sulphate present in the lysis buffer and the ionic detergent SDS added before extraction are ribonuclease inhibitors, albeit incomplete inhibitors. The cell lysis step is performed as quickly as possible at cold temperatures to decrease the possibility of RNA degradation. The extraction step depends on phenol and chloroform to act as denaturing agents which will inactivate endogenous ribonucleases. These precautions are intended to inhibit ribonuclease attack, but some cell types possess ribonuclease activity which is sufficiently high (e.g. pancreatic exocrine cells) that intact RNA cannot be prepared by this method. One alternative is to use the strong denaturing agent guanidinium isothiocyanate to prepare cytoplasmic RNA (see Section 6.1.4).

Another alternative is to use other ribonuclease inhibitors during the preparation of cytoplasmic RNA. For example, vanadyl ribonucleoside complexes (Life Technologies) inhibit many of the known ribonucleases, but do not interfere with most enzymatic reactions (15). Vanadyl ribonucleoside complexes should be used at a concentration of 10 mM during the cell lysis step.

Some protocols dictate adding urea and EDTA during extraction with phenol-chloroform (16). Urea is a strong denaturing agent and EDTA chelates Mg^{2+} ions required for the activity of some ribonucleases. The use of these agents is suggested for cells with a high content of ribonucleases (see the footnote to *Protocol 5*).

A16

Immunopathophysiological aspects of an emerging neonatal infectious disease induced by a bacterial superantigen

Naoto Takahashi,[1] Hidehito Kato,[2] Ken'ichi Imanishi,[2] Keishi Miwa,[3] Sadao Yamanami,[4] Hiroshi Nishida,[1] and Takehiko Uchiyama[2]

[1]Maternal and Perinatal Center, and
[2]Department of Microbiology and Immunology, Tokyo Women's Medical University School of Medicine, Tokyo, Japan
[3]Medical Devices and Diagnostics Research Laboratories, Toray Industries Inc., Ohtsu, Japan
[4]Neonatal Center, Kawaguchi Municipal Medical Center, Kawaguchi, Japan

Address correspondence to: Naoto Takahashi, Maternal and Perinatal Center, Tokyo Women's Medical University School of Medicine, 8-1 Kawadacho, Shinjuku-ku, Tokyo, Japan 162-8666.
Phone: 81-3-3355-3010; Fax: 81-3-5269-7345; E-mail: naoto-t@xa2.so-net.ne.jp.

Received for publication May 22, 2000, and accepted in revised form October 23, 2000.

We recently discovered an emerging neonatal infectious disease, neonatal toxic shock syndrome–like (TSS-like) exanthematous disease (NTED), which is induced by a superantigen, TSS toxin-1 (TSST-1), produced by methicillin-resistant *Staphylococcus aureus* (MRSA). Here, we analyzed the activation and the response of TSST-1–reactive Vβ2$^+$ T cells in NTED patients during the acute and recovery phases and in asymptomatic infants exposed to MRSA. In the acute phase, Vβ2$^+$ T cells were anergic to stimulation with TSST-1 and underwent marked expansion, but by 2 months after disease onset, their numbers had declined to about 10% of the control level. Although the percentage of Vβ2$^+$ T cells in the ten asymptomatic neonatal MRSA carriers was within the control range, these individuals could be divided into two groups on the basis of Vβ2$^+$ T-cell activation. Vβ2$^+$CD4$^+$ T cells from three of these infants (Group 1) highly expressed CD45RO and were anergic to TSST-1, whereas in the other seven asymptomatic neonatal MRSA carriers (Group 2), these cells expressed CD45RO at the control level and were highly responsive to stimulation with TSST-1. The serum anti–TSST-1 IgG Ab titer was negligible in the four NTED patients in the acute phase and the three asymptomatic neonatal MRSA carriers in Group 1, but it was high in the seven asymptomatic carriers in Group 2. We suggest that maternally derived anti–TSST-1 IgGs helps to suppress T-cell activation by TSST-1 and protects infants from developing NTED.

J. Clin. Invest. **106**:1409–1415 (2000).

Introduction

Several years ago, we saw a number of neonates who developed systemic exanthema, fever, low-positive serum C-reactive protein (CRP) values, and thrombocytopenia within the first week of life and described this disorder as a new disease entity (1, 2). Subsequently, several research groups, including our own, found that virtually all of the neonatal patients with this disease had been colonized by methicillin-resistant *Staphylococcus aureus* (MRSA) that produced selectively toxic shock syndrome (TSS) toxin-1 (TSST-1) (3–6), which has superantigenic activity (7, 8). This finding suggested a close relationship between this disease and TSS, which is caused by overactivation of TSST-1–reactive T cells (8–11). We found recently that Vβ2$^+$ T cells, which are the major TSST-1–reactive human T cells (12), were polyclonally expanded in the neonatal patients, and we named this disease neonatal TSS-like exanthematous disease (NTED) (13). NTED regressed spontaneously without antibiotic therapy in full-term neonates, but most preterm neonates developed severe symptoms (13). According to the responses to our questionnaires, neonates fulfilling the clinical criteria of NTED were observed in 25.7% (19/74) of major neonatal care units in Japan in 1995 and in 70.8% (63/89) in 1998, indicating that the incidence of NTED in Japan has been increasing. This finding is thought to be related to the conversion of MRSA into the TSST-1–producing type in Japan (14). There is apprehension that NTED will become a widespread disease throughout the world, because MRSA has been spreading on a global level (15, 16).

NTED, like TSS, is caused by the overactivation of TSST-1–reactive T cells (13). There are several immunological points that must be resolved to obtain clues that will allow us a comprehensive understanding of NTED and will enable the development of methods of prevention. First, a biphasic response consisting of a transient massive expansion and a subsequent protracted anergic and deleted state has been found to be induced in superantigen-reactive T-cell populations in experiments in mice (7, 8, 17–19). It remains to be elucidated whether the same response pattern is induced in NTED patients. Second, around 20% of the neonates in the neonatal

Table 1
Clinical profiles of the NTED patients, asymptomatic neonatal MRSA carriers, and MRSA-free neonates

	GA (wk)	BW (kg)	Postnatal day Fever onset	Exanthema manifested	Maximum[A] WBC ($10^3/\mu l$)	Lym	Minimum[A] Platelet ($10^3/\mu l$)	Maximum CRP (mg/l)	MRSA culture Swabs Nasal	Oral	Umbil	Stools
P1	38	2.9	2	4–6	14.4	5.0	98	19	+	–	+	+
P2	33	1.3	–	3–5	18.4	8.8	127	28	nd	+	+	+
P3	37	2.3	2	3–4	12.9	8.3	86	43	+	–	+	+
P4	39	2.9	2	3–4	16.2	11.5	80	36	nd	+	+	nd
Asymptomatic MRSA carriers												
Ca1	39	3.1	–	–	12.3	4.0	444	<3.0	+	–	+	+
Ca2	39	3.2	–	–	8.8	3.7	nd	<3.0	–	–	+	–
Ca3	38	3.7	–	–	8.0	2.1	288	<3.0	+	–	+	nd
Ca4	38	3.0	–	–	6.7	2.0	456	<3.0	+	–	+	+
Ca5	38	3.0	–	–	8.4	5.2	234	<3.0	+	–	+	nd
Ca6	38	2.9	–	–	6.5	1.7	245	<3.0	–	–	+	+
Ca7	38	2.6	–	–	7.8	4.1	502	<3.0	–	–	+	–
Ca8	40	3.7	–	–	9.5	5.4	439	<3.0	–	–	+	+
Ca9	40	4.2	–	–	9.6	5.6	402	<3.0	+	–	+	nd
Ca10	38	3.0	–	–	8.4	5.2	234	<3.0	–	–	+	nd
MRSA-free neonates (mean ± SD)[B]												
	38.6 ±1.1	2.9 ±0.2	–	–	8.2 ±1.5	5.7 ±0.9	446 ±115	<3.0	–	–	–	–

[A]The white blood cell counts and platelet counts were maximal and minimal, respectively, on day 2 or 3 after the onset of exanthema in the four NTED patients and were examined on postnatal day 5 in the asymptomatic MRSA carriers and MRSA-free neonates. [B]Mean ± SD in the eight MRSA-free neonates on postnatal day 5. GA, gestational age; BW, birth weight; Lym, lymphocyte count; +, MRSA isolated; –, MRSA not isolated; nd, not done.

care unit of Tokyo Women's Medical University Hospital are MRSA carriers, and 10% of the 20% have manifested symptoms of NTED. We speculated that T-cell activation by TSST-1 was induced in a certain proportion of asymptomatic neonatal MRSA carriers and that a large proportion of them was protected from the development of NTED by the transplacental transfer of anti–TSST-1 Ab of maternal origin.

In the present study we obtained evidence indicating that long-lasting immunological tolerance was induced in the $V\beta2^+$ T cells of the NTED patients, that activation of $V\beta2^+$ T cells occurred in a certain proportion of asymptomatic neonatal MRSA carriers, and that anti–TSST-1 IgG Ab of maternal origin plays a protective role in preventing the development of NTED. We discuss the implications of our findings in relation to the pathogenetic mechanism underlying NTED and its prevention.

Methods

Neonates examined in the present study. Four NTED patients, ten asymptomatic neonatal MRSA carriers and eight MRSA-free neonates were registered as the subjects of this study. The NTED patients had been admitted to the neonatal care unit of Tokyo Women's Medical University Hospital or Kawaguchi Municipal Medical Center and had been diagnosed on the basis of the clinical criteria established by us for NTED, erythema plus at least one of the following three manifestations: thrombocytopenia, a low-positive serum CRP value, and fever (1, 2, 13). Nasal, oropharyngeal and umbilical swabs, and stools, collected from neonates on postnatal day 3, were examined for the presence of MRSA to select asymptomatic MRSA-carriers and MRSA-free neonates. After obtaining the informed consent of their parents, peripheral blood samples were collected from the NTED patients in the acute phase and in the recovery phase and from the asymptomatic MRSA carriers and MRSA-free neonates on postnatal day 5.

Reagents and mAb's. FITC-conjugated MPB2D5 (anti-Vβ2), CH92 (anti-Vβ3), SFCI12T4D11 (anti-CD4), phycoerythrin-cyanin 5.1–conjugated (PC5-conjugated) UCHT1 (anti-CD3), 13B8.2 (anti-CD4), and B9.11 (anti-CD8) were purchased from Coulter Corp. (Hialeah, Florida, USA). Phycoerythrin-conjugated (PE-conjugated) UCHL1 (anti-CD45RO) and SK1 (anti-CD8) were purchased from Becton Dickinson Immunocytometry Systems (Mountain View, California, USA). TSST-1 and staphylococcal enterotoxin A (SEA) were purchased from Toxin Technology (Sarasota, Florida, USA). The RPMI-1640 culture medium used contained 10% FCS and 5 10^{-5} M 2-ME. The recombinant IL-2 used in the IL-2 assay was kindly provided by Takeda Chemical Industries (Kyoto, Japan).

Characterization of the immunological phenotypes of T cells by flow cytometry. PBMCs were isolated by Ficoll-Conray density-gradient centrifugation, as described previously (20, 21). To examine the percentage of $V\beta2^+$ T cells (reactive with TSST-1) or $V\beta3^+$ T cells (reactive with staphylococcal enterotoxin B; SEB) (7, 8), PBMCs were stained with several combinations of adequate PC5-, FITC-, or PE-conjugated mAb's and examined by three-color flow-cytometry analysis using a EPICS XL flow cytometer (Coulter Corp.), as described previously (20, 21).

Assay of superantigen-induced IL-2 production by mononuclear cells. Isolated PBMCs (2 10^5) were stimulated with 10 ng of TSST-1 or SEA per milliliter in 200-μl vol-

umes in round-bottom 96-well culture plates (Becton Dickinson, Franklin Lakes, New Jersey, USA) for various durations. IL-2 activity in the culture supernatants was determined by using IL-2–dependent CTLL-2 cells, as reported previously (20, 21). Data are shown as units of IL-2 per milliliter.

Measurement of anti–TSST-1 Ab's by ELISA. Titers of anti–TSST-1 Ab's (IgG and IgM) were measured by ELISA, as described previously (22). Briefly, serum diluted to 1:1000 and 1:100 for titration of IgG and IgM anti–TSST-1 Ab's, respectively, was applied to either TSST-1–precoated or noncoated plates, in duplicate. Peroxidase-conjugated rabbit anti-human IgG or IgM Ab's (Organo Teknika Corp., West Chester, Pennsylvania, USA) and tetramethyl benzidine (Sigma Chemical Co., St. Louis, Missouri, USA) were added to the plates, and the Ab titers were determined based on the OD at 450 nm. Data are shown as OD.

Statistical analysis. Statistical analysis was performed using the Mann-Whitney's *U*-test. *P* values less than 0.05 were considered significant.

Results

Clinical profiles and laboratory data of NTED patients, asymptomatic MRSA carriers, and MRSA-free neonates. The clinical profiles of the four NTED patients, ten asymptomatic MRSA carriers, and eight MRSA-free neonates examined are shown in Table 1. NTED patient P2 was admitted to the intensive care unit soon after birth due to being a preterm infant. The other NTED patients P1, P3, and P4, were transferred from the newborn nursery to the intensive care unit after the onset of NTED. Systemic exanthema, fever, low-positive serum CRP values, and thrombocytopenia were consistently observed in the full-term neonates, P1, P3, and P4. No fever was noted in the preterm neonate, P2, who exhibited apnea attacks and food intolerance and had symptomatic patent ductus arteriosus. While all three full-term neonates with NTED recovered spontaneously without any antibiotic therapy, the preterm neonate, P2, recovered after treatment with vancomycin. On day 2–3 after the onset of the exanthema, the white blood cell (WBC) and lymphocyte counts of all four NTED patients were high, and their platelet counts were low. With the exception of a slight increase in WBC count in one MRSA carrier, Ca1, the laboratory data in the ten asymptomatic neonatal MRSA carriers were almost the same as in the MRSA-free neonates. It is noteworthy that the umbilicus was a major site of colonization by TSST-1–producing MRSA. All asymptomatic MRSA carriers and MRSA-free neonates remained in the newborn nursery and were discharged from the hospital without any clinical manifestations. None of the ten asymptomatic MRSA carriers showed any symptoms during the first month of life.

Selective expansion and specific anergy induction in Vβ2⁺ T cells in acute-phase NTED patients. The immunological state of the four NTED patients was investigated. First, the peripheral blood mononuclear (PBM) T cells of the NTED patients and MRSA-free neonates were examined to determine the percentage of Vβ2⁺ T cells. The results are summarized in Table 2. The number of CD3⁺ T cells was increased in three of the four NTED patients, and the percentages of Vβ2⁺ CD4⁺ and Vβ2⁺ CD8⁺ T cells were significantly higher in the four NTED patients than in the eight MRSA-free neonates. A high percentage of these expanded Vβ2⁺ T cells in the NTED patients expressed CD45RO, whereas only a low or negligible percentage of the Vβ2⁺ T cells of the eight MRSA-free neonates expressed CD45RO. The percentages of Vβ3⁺CD4⁺ and Vβ3⁺CD8⁺ T cells of the NTED patients that are reactive with staphylococcal enterotoxin B (SEB) (7, 8) were low (Table 2), as reported previously (13). These results indicate that Vβ2⁺ T cells

Table 2
Expansion and anergy induction to TSST-1 in TCR Vβ2⁺ T cells from NTED patients in the acute phase

	Age (days)	T cells (10^3/μl)	CD4/CD8 ratio	Percentage of Vβ2+ T cells (CD45RO⁺)[B] CD4⁺	Vβ2+ T cells (CD45RO⁺) CD8⁺	Vβ3+ T cells (CD45RO⁺) CD4⁺	Vβ3+ T cells (CD45RO⁺) CD8⁺	Toxin	IL-2 production (U/ml)[C] Period of stimulation 8 h	24 h	48 h
NTED patients											
P1	5	5.5	2.9	27.2[A]	36.7	5.7	7.4	TSST-1	<0.1	<0.1	<0.1
				(86.0)[B]	(86.2)	(5.7)	(4.8)	SEA	1.0	5.3	16.2
P2	4	6.4	5.0	29.5	29.0	6.8	8.5	TSST-1	<0.1	<0.1	<0.1
				(96.7)	(69.7)	(5.7)	(2.8)	SEA	0.3	13.0	28.0
P3	4	4.3	4.6	25.4	26.4	nd	nd	TSST-1	1.7	0.6	<0.1
				(85.1)	(85.6)			SEA	1.8	9.5	20.0
P4	5	9.8	6.0	25.7	21.7	nd	nd	TSST-1	<0.1	0.8	2.0
				(57.1)	(44.2)			SEA	1.5	14.0	63.0
MRSA-free neonates (controls)[D]											
	5	4.4 ± 1.7	4.0 ± 2.0	11.5 ± 1.5	6.1 ± 0.9	5.3 ± 2.7	4.9 ± 2.7	TSST-1	2.7 ± 0.9	33.6 ± 5.2	76.3 ± 13.4
				(5.1 ± 2.5)	(1.0 ± 1.1)	(6.2 ± 1.8)	(0.8 ± 0.8)	SEA	2.8 ± 0.6	40.1 ± 8.8	86.0 ± 55.4

[A]Percentage of Vβ2⁺CD4⁺ and Vβ2⁺CD8⁺ T cells or Vβ3⁺CD4⁺ and Vβ3⁺CD8⁺ T cells among PBM T cells. [B]Percentage of the CD45RO⁺ fraction among the Vβ2⁺CD4⁺ and Vβ2⁺CD8⁺ T cells or Vβ3⁺CD4⁺ and Vβ3⁺CD8⁺ T cells. [C]PBMCs (2 10^5/culture) were stimulated in vitro with 10 ng of TSST-1 or SEA per milliliter for indicated periods, and the culture supernatants were assayed for IL-2 activity. [D]Mean ± SD in the eight MRSA-free neonates on postnatal day 5. nd, not done.

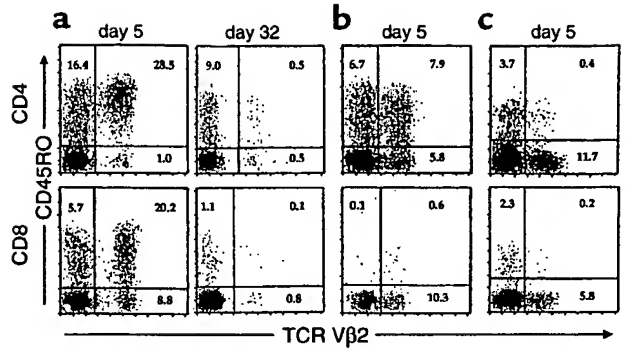


Figure 1
Expression of T-cell receptor Vβ2 versus CD45RO on CD4⁺ and CD8⁺ T cells obtained from an NTED patient in the acute and recovery phases, an asymptomatic MRSA carrier, and an MRSA-free neonate. The percentage of Vβ2⁺ T cells and the expression levels of CD45RO by Vβ2⁺CD4⁺ and Vβ2⁺CD8⁺ T cells were examined in the preterm NTED patient P2 on postnatal days 4 and 32 (a), in the asymptomatic neonatal MRSA carrier Ca1 on postnatal day 5 (b), and in an MRSA-free neonate on postnatal day 5 (c). The numbers are the percentages of stained cells in each area.

exhibited selective activation and expansion induced by TSST-1 in the NTED patients, irrespective of the CD4⁺ or CD8⁺ T-cell subsets. The flow-cytometric findings for the expansion of Vβ2⁺ T cells expressing CD45RO in patient P2 are shown in Figure 1a.

Second, to investigate whether the expanded TSST-1–reactive T cells retained their ability to respond to stimulation with TSST-1 in the NTED patients, we examined IL-2 production by PBM T cells from the NTED patients and MRSA-free neonates in response to in vitro stimulation with TSST-1 or an unrelated superantigen, SEA. As shown in Table 2, PBM T cells from MRSA-free neonates exhibited marked IL-2 production in response to stimulation with either TSST-1 or SEA. By contrast, the PBM T cells from the NTED patients exhibited no or only a minimum level of IL-2 production in response to stimulation with TSST-1, but exhibited IL-2 production that was substantial, although slightly lower than in the MRSA-free neonates, in response to stimulation with SEA. The results indicate that the expanded TSST-1–reactive Vβ2⁺ T cells of the NTED patients were specifically anergic to TSST-1, as seen in the superantigen-reactive T cells of mice injected with these superantigens (17–19).

Protracted deletion of Vβ2⁺ T cells in NTED patients. We monitored the Vβ2⁺ T cells in the peripheral blood of the four NTED patients for certain periods after the onset of the disease to determine their fate. The results are shown in Figure 2. The percentages of Vβ2⁺CD4⁺ T cells and Vβ2⁺CD8⁺ T cells in the four patients were quite high in the acute phase, as shown above, but decreased to around the levels of the MRSA-free neonates on postnatal day 5 (the control) within 10 days and to very low levels, around 10% of the control level, by 1 or 2 months after the onset of disease (Figure 2, a and b). The 5-month follow-up examination of patient P1 revealed 50% recovery of the deleted Vβ2⁺ T cells to the control level. The percentages of Vβ2⁺CD4⁺ and Vβ2⁺CD8⁺ T cells were almost the same in the eight MRSA-free neonates on postnatal day 5, the one MRSA-free neonate on postnatal day 39, and the seven healthy adults, respectively (Figure 2, a and b), indicating that the levels of Vβ2⁺CD4⁺ and Vβ2⁺CD8⁺ T cells do not change much with age in healthy individuals. These findings indicate that a biphasic response consisting of transient expansion and subsequent specific deletion was induced in the Vβ2⁺ T cells of the NTED patients.

Immunological state of TSST-1–reactive T cells in asymptomatic neonatal MRSA carriers. The immunological state of the asymptomatic neonatal MRSA carriers was investigated. First, PBM T cells from the ten asymptomatic neonatal MRSA carriers were examined for the expression of Vβ2 and CD45RO on postnatal day 5. Although the percentage of their Vβ2⁺ T cells was within the normal range for both CD4⁺ and CD8⁺ T cells, we found increased expression of CD45RO by Vβ2⁺CD4⁺ T cells in three (Ca1–Ca3) of the ten neonatal carriers (Table 3), suggesting that Vβ2⁺CD4⁺ T cells were acti-

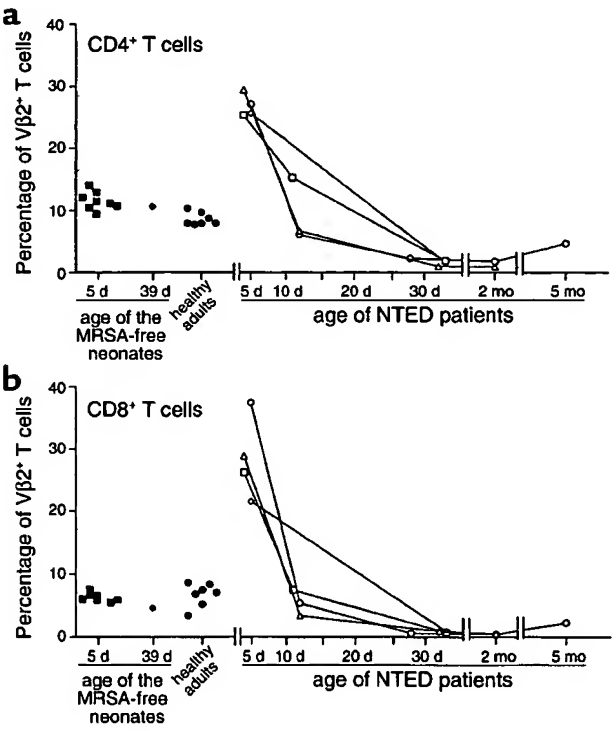


Figure 2
Fate of TCR Vβ2⁺ T cells in NTED patients. The four NTED patients in the acute and recovery phases, nine MRSA-free neonates on postnatal day 5 or 39, and seven healthy adults were examined for the percentage of Vβ2⁺CD4⁺ T cells (a) and Vβ2⁺CD8⁺ T cells (b) among PBMCs. NTED patients P1 (open circles), P2 (open triangles), P3 (open squares), and P4 (open diamonds) refer to the same cases as in Tables 1 and 2. Filled squares, MRSA-free neonates on postnatal day 5; filled diamonds, MRSA-free neonates on postnatal day 39; filled circles, healthy adults.

Table 3
Immunologic state of the asymptomatic neonatal MRSA carriers

MRSA carriers	T cells (10^3/μl)	CD4 to CD8 ratio	Percentage of Vβ2⁺ T cells[A] (CD45RO⁺ fraction)[B] CD4⁺	CD8⁺	IL-2 production (U/ml)[C] Period of stimulation toxin	8 h	24 h
Group 1							
Ca1	2.9	2.2	13.7[A] (57.6)[B]	10.9 (5.1)	TSST-1	0.6	1.6
					SEA	1.4	32.0
Ca2	2.6	3	11.1 (55.8)	9.1 (5.7)	TSST-1	0.7	2.0
					SEA	1.0	23.0
Ca3	0.9	3.2	15.0 (27.5)	6.8 (4.4)	TSST-1	1.4	7.0
					SEA	2.6	15.0
Group 2							
Ca4	1.6	6.7	9.9 (10.2)	5.5 (0.1)	TSST-1	0.9	12.0
					SEA	1.0	25.0
Ca5	3.9	3.9	14.7 (4.1)	6.8 (4.3)	TSST-1	3.6	20.0
					SEA	4.5	16.2
Ca6	1.3	3.6	13.7 (6.1)	7.6 (0.3)	TSST-1	1.1	23.0
					SEA	0.6	25.0
Ca7	3.2	5.9	7.5 (3.9)	6.7 (0.0)	TSST-1	nd	nd
					SEA	nd	nd
Ca8	4.1	5.4	10.5 (3.2)	6.6 (0.9)	TSST-1	nd	nd
					SEA	nd	nd
Ca9	4.3	1.8	9.4 (4.6)	5.9 (0.0)	TSST-1	nd	nd
					SEA	nd	nd
Ca10	3.3	2.9	10.7 (1.9)	5.9 (1.8)	TSST-1	nd	nd
					SEA	nd	nd
MRSA-free neonates[D]							
	4.4 ± 1.7	4.0 ± 2.0	11.5 ± 1.5	6.1 ± 0.9	TSST-1	2.7 ± 0.9	33.6 ± 5.2
			(5.1 ± 2.5)	(1.0 ± 1.1)	SEA	2.8 ± 0.6	40.1 ± 8.8

Ten asymptomatic neonatal MRSA carriers were examined on postnatal day 5 for expression of CD45RO by Vβ2⁺ PBM T cells and TSST-1–induced IL-2 production by PBMCs. They were divided arbitrarily into two groups on the basis of activation level of Vβ2⁺CD4⁺ T cells in three cases (Ca1–Ca3) with high CD45RO expression by Vβ2⁺CD4⁺ T cells and low TSST-1–induced IL-2 production by PBMCs (Group 1) and seven cases (Ca4–Ca10) with low CD45RO expression by Vβ2⁺CD4⁺ T cells and high IL-2 production (Group 2). Although TSST-1–induced IL-2 production was not examined in four neonates (Ca7–Ca10), we classified them into Group 2 on the basis of their low expression of CD45RO by Vβ2⁺ CD4⁺ T cells. [A]Percentage of Vβ2⁺ T cells among PBM CD4⁺ or CD8⁺ T cells. [B]Percentage of CD45RO⁺ cells among Vβ2⁺CD4⁺ and Vβ2⁺CD8⁺ T cells ($P = 0.017$ between Groups 1 and 2). [C]PBMCs (2×10^5/culture) were stimulated in vitro with 10 ng of TSST-1 or SEA per milliliter for 8 hours or 24 hours, and the culture supernatants were assayed for IL-2 activity. [D]Mean ± SD in eight MRSA-free neonates on postnatal day 5. nd, not done.

vated by TSST-1 in these neonates and intact in the other seven neonates. The flow-cytometric findings in Ca1 are shown in Figure 1b.

We then examined IL-2 production by PBM T cells in response to in vitro stimulation with TSST-1 and SEA in the above three (Ca1–Ca3) of the ten asymptomatic neonatal MRSA carriers and in three (Ca4–Ca6) of the other seven carriers. The PBM T cells of neonates Ca1–Ca3 did not produce IL-2 in response to stimulation with TSST-1 but responded normally to stimulation with SEA. By contrast, the PBM T cells from neonates Ca4-Ca6 responded normally to both TSST-1 and SEA (Table 3). The IL-2 response of neonates Ca7–Ca10 was not examined. The results indicated that the Vβ2⁺CD4⁺ T cells of neonates Ca1–Ca3 were anergic to TSST-1, but that the Vβ2⁺CD4⁺ T cells of neonates Ca4–Ca6 were intact. The PBM T cells of neonates Ca7–Ca10 presumably responded normally to both TSST-1, and SEA because the level of expression of CD45RO by their Vβ2⁺CD4⁺ T cells was within the control range (Table 3).

The results indicated that the Vβ2⁺CD4⁺ T cells of three (Ca1–Ca3) of the ten asymptomatic neonatal MRSA carriers had been activated by TSST-1 and suggested that they were not activated by TSST-1 in the other seven, but were intact. We therefore arbitrarily divided the ten asymptomatic neonatal MRSA carriers into two groups on the basis of Vβ2⁺ T-cell activation: Group 1, neonatal carriers (Ca1–Ca3) with Vβ2⁺CD4⁺ T cells activated by TSST-1; and Group 2, carriers (Ca4–Ca10) with intact Vβ2⁺CD4⁺ T cells.

Protective role of anti–TSST-1 Ab of maternal origin against the influence of TSST-1. Neonates are generally protected against various infectious agents by specific IgG Ab's transferred transplacentally from their mothers (23). As anti-TSST-1 Ab's are known to play a protective role against the development of TSS in adults (24), it seems likely that anti-TSST-1 Ab's transferred placentally from their mothers play a role in protecting neonates from developing NTED. We determined the titers of anti-TSST-1 IgG and IgM Ab's in the serum of the NTED patients in the acute and recovery phases, the asymptomatic neonatal MRSA carriers, and the MRSA-free neonates on postnatal day 5 (Figure 3). In the four neonates with NTED, the serum anti-TSST-1 IgG Ab titer was negligible in the acute phase of the disease, but

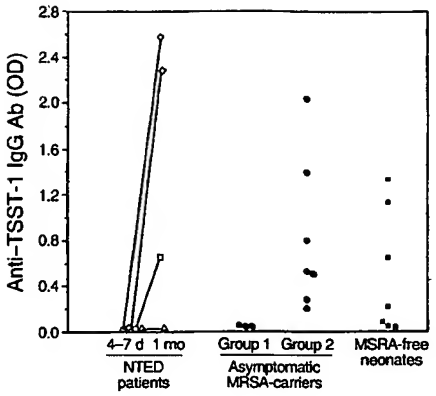


Figure 3

Titers of anti-TSST-1 IgG Ab in NTED patients, asymptomatic MRSA carriers, and MRSA-free neonates. Anti-TSST-1 IgG Ab titers were examined by ELISA in the serum of the four NTED patients P1 (open circles), P2 (open triangles), P3 (open squares), P4 (open diamonds) in the acute and recovery phases, ten asymptomatic neonatal MRSA carriers on postnatal day 5 (filled circles), and seven MRSA-free neonates on postnatal day 5 (filled squares). They were determined based on the OD at 450 nm.

increased within 1 month in the patients P1, P3, and P4, but not in the preterm patient P2. In the asymptomatic neonatal MRSA carriers, the anti-TSST-1 IgG Ab titer was almost negligible in the three Group 1 neonates with $V\beta2^+CD4^+$ T cells activated by TSST-1 (Table 3), but was high (0.2 or more) in the Group 2 neonates without $V\beta2^+CD4^+$ T cells activated by TSST-1. The anti-TSST-1 Ab titer was low (less than 0.2) in three of the eight MRSA-free neonates examined and high (more than 0.2) in the other five. The anti-TSST-1 IgM Ab titer was negligible soon after their birth (4–7 days) in all four NTED patients, the asymptomatic neonatal MRSA carriers, and the MRSA-free neonates (data not shown). At 1 month of life, the anti-TSST-1 IgM Ab titer had increased only slightly, from less than 0.01 to 0.02–0.03, in three NTED patients.

Discussion

In the present study, we examined several immunological aspects of a newly discovered neonatal disease, NTED, induced by superantigen TSST-1 in order to obtain clues that could lead to a comprehensive understanding of the pathogenetic mechanism underlying NTED and the development of methods to prevent the disease. The results clarified the immunological events that occurred in TSST-1–reactive human T cells and the protective role of anti-TSST-1 IgG Ab's transferred transplacentally against the development of NTED.

The TSST-1–reactive $V\beta2^+$ T cells that exhibited expansion in the acute phase of NTED were found to be anergic to TSST-1 (Table 2), and the $V\beta2^+$ T cells in the expanded state were subsequently eliminated to around 10% of the control level by 1–2 months after the onset of disease (Figure 2), indicating that a response pattern similar to that in mice injected with bacterial superantigens (7, 8, 17–19) was also induced in human neonates exposed to TSST-1. Recovery of the deleted $V\beta2^+$ T cells to 50% of the control level occurred at around 5 months (Figure 2), suggesting that 6 months or more are required for the recovery of deleted clones to normal levels in neonates. As in the NTED patients, expansion of $V\beta2^+$ T cells was found previously in adult TSS patients (ref. 11 and our unpublished data). The deletion of the expanded $V\beta^+$ T cells, however, was not as profound or rapid in the adult TSS patients; the $V\beta/C\alpha$ ratio of the $V\beta2^+$ examined by the PCR method in two blood samples drawn 25 and 50 days after the onset of symptoms was still higher than that in the control (11). We think that the implications of the results of these studies in TSS in adults and NTED are important in terms of T-cell maturity in the neonatal period and the outcome of NTED.

According to Burnet's clonal selection theory (25), elimination of self-reactive lymphocytes occurs early in life, and the results of many experiments, including those of Billingham et al. (26), mainly using mice, have supported his concept and given rise to the notion that T cells in the neonatal period are intrinsically susceptible to anergy induction. This view has been refuted by reports suggesting that the T cells of neonatal and mature individuals are not qualitatively different, as reviewed by Stockinger (27). Recently, however, we observed that human $CD1a^-$ $CD4^+$ T cells in the final stage of maturation in the thymus and cord-blood $CD4^+$ T cells that supposedly had migrated recently from the thymus were susceptible to anergy induction by in vitro stimulation with TSST-1, whereas adult PBM $CD4^+$ T cells were resistant (20, 21), indicating the immaturity of human T cells during the neonatal period. On the basis of the results of the studies described above, we think that the rapid elimination of TSST-1–reactive $V\beta2^+$ T cells after their transient expansion in NTED patients is a reflection of the intrinsically immature state of the T cells resident in neonates. Because NTED is caused by overactivation of TSST-1–reactive T cells, mainly $V\beta2^+$ T cells (13), the rapid recovery from the illness without any complications in most full-term NTED patients (1, 2, 13) seems to be largely attributable to this immaturity of the T cells, the high susceptibility to anergy induction, and rapid deletion of $V\beta2^+$ T cells in the early neonatal period. The multiorgan failure seen in mature TSS patients could be caused by the persistent activated state of the $V\beta2^+$ T cells.

The percentages of $V\beta2^+CD4^+$ T cells and $V\beta2^+CD8^+$ T cells in the ten asymptomatic neonatal MRSA carriers examined were within the normal range (Table 3), however, they could be divided into two groups, Group 1 and Group 2, on the basis of the TSST-1–induced activation of their $V\beta2^+$ T cells (Table 3). The $V\beta2^+$ T cells in Group 1 neonates were activated by TSST-1, but they were intact and not activated in Group 2 neonates. These results suggest that the TSST-1–reactive T cells are activated by TSST-1 in

about 30% of neonatal MRSA-carriers, although the number of neonates examined was too low to evaluate the these findings statistically.

A question arises as to what factors divided the neonatal MRSA carriers into NTED patients and the Group 1 and Group 2 asymptomatic neonatal MRSA carriers. The view that NTED is caused by overactivation of TSST-1-reactive T cells (13) suggests that the level of activation of Vβ2$^+$ T cells governs the development of NTED. We think that the level of activation of Vβ2$^+$ T cells can be determined mainly by the amount of TSST-1 absorbed and anti-TSST-1 IgG Ab that can neutralize the superantigenic activity of TSST-1 in neonates. The level of Vβ2$^+$ T-cell activation in the four NTED patients was clearly higher than in the three Group 1 asymptomatic neonatal MRSA carriers, as shown in Table 2, Table 3, and Figure 1. Another clue as to the answer to the question was obtained from the analysis of the anti-TSST-1 IgG Ab titer in the NTED patients and the asymptomatic neonatal MRSA carriers. The results showed that the serum anti-TSST-1 IgG Ab titer was negligible in all four NTED patients and Group 1 neonates, but high in the Group 2 neonates in the early days after birth (Figure 3). We presume that higher amounts of TSST-1 were absorbed in the NTED patients than in the Group 1 neonates.

Anti-TSST-1 IgG Ab was found to be effective in blocking the superantigenic activity of TSST-1 and protecting against the development of NTED, as shown in Figure 3 and discussed above. This finding indicates that transfer of high amounts of anti-TSST-1 IgG Ab from mothers to their children through the placenta are effective in protecting against the development of NTED. The effectiveness of vaccination in preventing abnormal superantigen-induced reactions by a superantigen that has lost its superantigenic activity has been studied in animal experiments (28). Vaccination of pregnant women with attenuated TSST-1 may be one means of preventing the development of NTED in their children.

There are several questions as to whether TSST-1-reactive T cells of NTED patients retain long-term memory against TSST-1 that can be seen in the anergy induction to stimulation with TSST-1 and whether the TSST-1-reactive T cells in an activated state in the Group 1 neonates normalize with time. Does complete deletion of Vβ2$^+$ T cells occur in the recovery phase in NTED patients, when the patients are exposed to a high amounts of TSST-1? These questions are left to be clarified in future studies.

Acknowledgments

This work was supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Ministry of Health and Welfare of Japan, and The Mother and Child Health Foundation. The authors thank M. Maruyama for identification of the MRSA carriers and I. Sakuma for collecting the blood specimens.

1. Takahashi, N., et al. 1995. A new exanthematous disease in newborn infants. *Acta Neonatologica Japonica.* **31**:371-377.
2. Takahashi, N., and Nishida, H. 1997. A new exanthematous disease with thrombocytopenia in newborn infants. *Arch. Dis. Child.* **77**:F79.
3. Makimoto, A., Kubo, M., and Kawakami, K. 1996. Neonatal thrombocytopenia with skin eruption, probably caused by exotoxin-producing methicillin-resistant *Staphylococcus aureus* (MRSA): clinical evaluation in 14 cases. *Journal of the Japan Pediatric Society.* **100**:609-615.
4. Matsuo, Y., et al. 1997. Clinical studies of early neonatal exanthematous disease with thrombocytopenia: in relation to methicillin resistant Staphylococcus aureus (MRSA). *J. Jpn. Soc. for Premature Newborn Med.* **9**:86-93.
5. Okada, T., Furukawa, S., Satomura, S., Hayabuchi, Y., and Ohta, A. 1997. Bacterial examinations of a new exanthematous disease in newborn infants. *Journal of the Japan Pediatric Society.* **101**:631-637.
6. Sakata, Y., et al. 1997. Exanthema of the newborn related to toxic shock syndrome toxin-1 (TSST-1). *Japanese Journal of Obstetrical, Gynecological and Neonatal Hematology.* **7**:146-150.
7. Kotzin, B.L., Leung, D.Y., Kappler, J., and Marrack, P. 1993. Superantigens and their potential role in human disease. *Adv. Immunol.* **54**:99-166.
8. Uchiyama, T., Yan, X.-J., Imanishi, K., and Yagi, J. 1994. Bacterial superantigens: mechanism of T cell activation by the superantigens and their role in the pathogenesis of infectious diseases. *Microbiol. Immunol.* **38**:245-256.
9. Uchiyama, T., et al. 1987. Study of the biological activities of toxic shock syndrome toxin-1: induction of the proliferative response and interleukin 2 production by T cells from human peripheral mononuclear cells stimulated with the toxin. *Clin. Exp. Immunol.* **68**:638-647.
10. Uchiyama, T., et al. 1989. Activation of human T cells by toxic shock syndrome toxin-1: the toxin binding structures expressed on human lymphoid cells acting as accessory cells are HLA class II molecules. *Eur. J. Immunol.* **17**:1803-1809.
11. Choi, Y., et al. 1990. Selective expansion of T cells expressing Vβ2 in toxic shock syndrome. *J. Exp. Med.* **172**:981-984.
12. Choi, Y., et al. 1989. Interaction of Staphylococcus aureus toxin "superantigens" with human T cells. *Proc. Natl. Acad. Sci. USA.* **86**:8941-8945.
13. Takahashi, N., et al. 1998. Exanthematous disease induced by toxic shock syndrome toxin 1 in the early neonatal period. *Lancet.* **351**:1614-1619.
14. Cui, L., Hanaki, H., and Hiramatsu, K. 1998. The epidemiology of MRSA producing TSST-1 and the influence of serum on the production of TSST-1 [in Japanese]. *Japanese Journal of Bacteriology.* **53**:155. (Abstr.)
15. Ayliffe, G.A. 1997. The progressive intercontinental spread of methicillin-resistant Staphylococcus aureus. *Clin. Infect. Dis.* **24**(Suppl. 1):S74-S79.
16. Archer, G.L. 1998. *Staphylococcus aureus*: a well-armed pathogen. *Clin. Infect. Dis.* **26**:1179-1181.
17. Kawabe, Y., and Ochi, A. 1990. Selective anergy of Vβ8$^+$, CD4$^+$ T cells in Staphylococcus enterotoxin B-primed mice. *J. Exp. Med.* **172**:1065-1070.
18. Rellahan, B.L., Jones, L.A., Kruisbeek, A.M., Fry, A.M., and Matis, L.A. 1990. In vivo induction of anergy in peripheral Vβ8+ T cells by staphylococcal enterotoxin B. *J. Exp. Med.* **172**:1091-1100.
19. Kuroda, K., et al. 1996. Implantation of IL-2-containing osmotic pump prolongs the survival of superantigen-reactive T cells expanded in mice injected with bacterial superantigen. *J. Immunol.* **157**:1422-1431.
20. Takahashi, N., Imanishi, K., Nishida, H., and Uchiyama, T. 1995. Evidence for immunologic immaturity of cord blood T cells. Cord blood T cells are susceptible to tolerance induction to in vitro stimulation with a superantigen. *J. Immunol.* **155**:5213-5219.
21. Imanishi, K., et al. 1998. Post-thymic maturation of migrating thymic single positive T cells: thymic CD1a$^-$ CD4$^+$ T cells are more susceptible to anergy induction by toxic shock syndrome toxin-1 than cord blood CD4$^+$ T cells. *J. Immunol.* **160**:112-119.
22. Terai, M., et al. 1995. The absence of evidence of staphylococcal toxin involvement in the pathogenesis of Kawasaki disease. *J. Infect. Dis.* **172**:558-561.
23. Wilson, C.B. 1990. Developmental immunology and role of host defenses in neonatal susceptibility. In *Infectious diseases of the fetus and newborn infant.* 3rd edition. J.S. Remington and J.O. Klein, editors. W.B. Saunders Co. Philadelphia, Pennsylvania, USA. 17-67.
24. Vergerout, J., et al. 1983. Prevalence of serum antibody to staphylococcal enterotoxin F among Wisconsin residents: implications for toxic shock syndrome. *J. Infect. Dis.* **148**:692-698.
25. Burnet, F.M. 1959. *The clonal selection theory of acquired immunity.* Cambridge University Press. Cambridge, United Kingdom. 208 pp.
26. Billingham, R.E., Brent, L., and Medawar, P.B. 1953. 'Actively acquired tolerance' of foreign cells. *Nature.* **172**:603-606.
27. Stockinger, B. 1996. Neonatal tolerance mysteries solved. *Immunol. Today.* **17**:249.
28. Bavari, S., Dyas, B., and Ulrich, R. 1996. Superantigen vaccines: a comparative study of genetically attenuated receptor-binding mutants of staphylococcal enterotoxin A. *J. Infect. Dis.* **174**:338-345.

A17

Clin Exp Immunol 1998; 114:94–101

Increased nitric oxide (NO) production by antigen-presenting dendritic cells is responsible for low allogeneic mixed leucocyte reaction (MLR) in primary biliary cirrhosis (PBC)

K. YAMAMOTO, SK. MD. FAZLE AKBAR, T. MASUMOTO & M. ONJI *Third Department of Internal Medicine, Ehime University School of Medicine, Ehime, Japan*

(Accepted for publication 2 July 1998)

SUMMARY

The levels of blastogenesis in allogeneic MLR containing T cells from one normal volunteer and irradiated dendritic cells from 29 patients with PBC, 17 patients with chronic hepatitis type C (CH-C) and 22 allogeneic normal controls were compared to see if there is any role of antigen-presenting cells (APC) in the pathogenesis of PBC. The stimulatory capacity of dendritic cells from PBC was significantly lower compared with that of dendritic cells from CH-C ($P < 0{\cdot}05$) and normal controls ($P < 0{\cdot}05$), which could not be attributable either to the levels of expression of surface molecules, such as HLA-DR and CD86 on dendritic cells, or to the levels of cytokines, such as IL-10 and IL-12. Significantly higher levels of NO were seen in the allogeneic MLR supernatants containing dendritic cells from PBC compared with the supernatants from cultures containing dendritic cells from CH-C ($P < 0{\cdot}001$) or normal controls ($P < 0{\cdot}001$). Moreover, dendritic cells from PBC produced 10 times more NO compared with dendritic cells from CH-C and normal controls ($21{\cdot}9 \pm 2{\cdot}8$ μM *versus* $1{\cdot}6 \pm 0{\cdot}3$ μM and $1{\cdot}6 \pm 0{\cdot}3$ μM, respectively; $P < 0{\cdot}001$). The addition of N^G-monomethyl-L-arginine monoacetate (L-NMMA), a known inhibitor of NO in allogeneic MLR containing dendritic cells from PBC, resulted in a significant decrease of NO and increase of blastogenesis. The selective impairment of dendritic cell function, increased production of NO by dendritic cells and restoration of blastogenesis using NO inhibitor in PBC have suggested a role for NO and dysfunction of dendritic cells in the pathogenesis of PBC. This inspires optimism that modulating the function of dendritic cells and controlling NO production, an improved therapeutic approach, might be planned for PBC.

Keywords dendritic cells nitric oxide primary biliary cirrhosis

INTRODUCTION

PBC is characterized by chronic inflammation and destruction of intrahepatic bile ducts [1]. The frequent association of PBC with other autoimmune syndromes [2,3], induction of hepatic inflammation in severe combined immunodeficient mice infused with mononuclear cells from patients with PBC [4] and the observation that a syndrome resembling PBC occurs in hepatic allografts [5] and in graft-*versus*-host disease [6] emphasize the role of abnormal host immune responses in PBC. Immunoregulatory mechanisms which normally inhibit potentially autodestructive immune processes and protect the host from the occurrence of autoimmune diseases are thought to be defective in PBC because a decrease in the functional capability of lymphocytes, such as decreased blastogenesis in autologous MLR [7,8], decreased proliferation of $CD4^+$ T cells [9], decreased production of cytokines such as lymphotoxin, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) by T lymphocytes [10], and defective IL-2 responsiveness of T cells [11] has been reported in PBC, in spite of the presence of increased numbers of activated lymphocytes [12]. These indicate that there might be a dysfunction of immune regulatory cells, such as antigen-presenting cells (APC) in PBC, because the major pathway of T lymphocyte activation is through the interaction between T cells and APC, the activated T lymphocytes are the final product of this complex series of interactions, and the functional capability of activated lymphocytes is dependent on the second signal delivered mainly by antigen-presenting dendritic cells (DC) [13].

The abnormal activation of DC has been implicated in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) [14–16], and

Correspondence: Kazuhisa Yamamoto, Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan.

Table 1. Age, sex distribution and clinical profile of subjects

	PBC	Normal controls	CH-C
Number	29	22	17
Age (years)	55·0 ± 2·2	44·0 ± 3·0	55·4 ± 3·3
Sex (M:F)	3:26	12:10	9:8
ALT (9–37 U/l)*	47·8 ± 6·6	26·9 ± 2·5	53·8 ± 8·2
AST (3–49 U/l)	55·0 ± 10·7	31·3 ± 4·2	66·8 ± 12·2
IgM (35–220 mg/dl)	430·0 ± 60·0	ND	110·5 ± 0·5
ALP (57–194 U/l)	358·0 ± 36·0	156·7 ± 19·9	167·4 ± 15·2
Platlet ($10^{-4}/\mu l$)	23·2 ± 1·8	27·4 ± 2·6	17·0 ± 1·1

Data are shown as mean ± s.e.m. ND, Not done.
CH-C, Chronic hepatitis type C.
* Figures in parentheses indicate normal range.

the presence of DC has been shown immunohistochemically in the vicinity of damaged bile ducts in patients with PBC [17]. All this circumstantial evidence indicates a possible role of DC in PBC and justifies a thorough characterization of DC in PBC, but there is a paucity of information regarding the function of DC in PBC. This has mainly been due to the technical limitation of isolating the trace population of DC from peripheral blood. The recent advancements in enriching DC from peripheral blood by using cytokines [18] led us to undertake the present study to evaluate the function of DC in patients with PBC. The levels of blastogenesis of lymphocytes shown by incorporation of ^{3}H-thymidine (^{3}H-TdR) were significantly lower in allogeneic MLR containing DC from PBC compared with that in allogeneic MLR containing DC from chronic hepatitis type C (CH-C) and normal controls, and thus indicated decreased stimulatory capacity of DC from PBC compared with that of DC from CH-C and normal controls, because the entire T cell population in allogeneic MLR was isolated from one single normal volunteer. The mechanism underlying the low stimulatory capacity of DC in PBC was studied from the expression of surface antigens on DC, from the capacity of DC to induce and to produce immunoregulatory cytokines, and by estimating the production of NO by DC. The significance of increased production of NO by DC in PBC is discussed to find the clinical implications of these observations to develop a new therapeutic approach for PBC.

PATIENTS AND METHODS

Subjects

DC were collected from a total of 29 patients with PBC, 22 healthy normal controls, and 17 patients with CH-C. All patients with PBC, attending the 3rd Department of Internal Medicine, Ehime University School of Medicine (Ehime, Japan) between 1996 and 1998 were enrolled in the study. The normal controls included volunteers with no history of liver disease. Informed consent was obtained from all patients and normal volunteers after explaining the nature and purpose of the study.

The age, sex and clinical profile of patients and controls are given in Table 1. In seven patients with PBC (age 52·4 ± 2·2 years; one male, six females) and seven normal controls (age 31·3 ± 2·2 years; seven males), DC were isolated from peripheral blood by negative selection method. In 22 patients with PBC (age 55·7 ± 2·5 years; two males, 20 females), 17 patients with CH-C (age 55·4 ± 3·3 years; nine males, eight females), and 15 normal controls (age 49·1 ± 3·1 years; five males, 10 females), the DC from peripheral blood were enriched by cytokines. There was a female predominance in PBC but there was no significant difference with regard to age distribution. Histopathological grading showed that 20, three and six patients belonged to Scheuer stages 1, 2 and 3, respectively, 24 were asymptomatic and five were symptomatic. All patients had clinical and biochemical features suggestive of the respective diagnoses and the final diagnoses were confirmed by histopathology [19]. All patients with PBC and CH-C had not been taking steroids for the last 6 months before collection of blood.

T cells used in the entire experiment were collected from a single normal volunteer (male, age 33 years) who had no previous history of liver disease and was not taking any drug during the experiment. Peripheral blood counts, such as erythrocyte counts, leucocyte counts, platelet counts, Hb%, and number of lymphocytes, were periodically checked and did not show any significant change during the entire experimental period of 12 months. Written, informed consent was taken from the T cell donor.

HLA typing was done in the T cell donor, whose HLA-DR B1 typing revealed HLA-DR1 as 0404 and HLA-DR2 as 1502. HLA typing in PBC patients showed only one patient with HLA-DR2 of 1502. In all other cases, there was no identical HLA type between patients with PBC and the T cell donor. HLA typing was not done in patients with CH-C or normal controls.

Cell isolation

Isolation of T cells. All T cell populations in this experiment were collected from a single normal volunteer. Peripheral blood mononuclear cells (PBMC) were collected from interface by centrifuging heparinized blood on Ficoll–Conray ($P = 1{\cdot}077$). Initially, the T lymphocytes were enriched from PBMC by rosetting T cells with 2-amino ethylisothiouronium bromide (AET; Sigma Chemical Co., St Louis, MO)-treated sheep erythrocytes, as described. In most of the experiments, purified populations of T cells were collected using a T cell recovery kit (Cellect; Biotex Labs Inc., Edmonton, Canada) in which PBMC were depleted of B cell populations by allowing them to pass through the affinity column containing polyclonal goat anti-human IgG (H + L). The purity of T cell populations was >95% as confirmed by staining with FITC-conjugated anti-CD3 in a flow cytometer.

Isolation of dendritic cells. DC populations were enriched from peripheral blood using two procedures, enrichment by negative selection and enrichment by cytokines.

Enrichment of DC by negative selection

Enriched populations of DC were isolated from 14 cases (seven patients with PBC and seven normal controls) by depleting other cell populations, exactly as described by Freudenthal & Steinman [20]. Briefly, heparinized blood was layered on Ficoll–Conray and centrifuged at 1000 *g* for 20 min at 21°C. PBMC were harvested from the interface. T lymphocytes were separated by rosetting at 4°C with AET-treated sheep erythrocytes. The ER^- fraction was incubated on 100-mm plastic dishes at 37°C in presence of 5% CO_2. The non-adherent cells after 36 h of adherence were panned twice on a bacteriological dish coated with human γ-globulin (Wako Pure Chemical Industries Ltd, Osaka, Japan) to deplete Fc receptor-bearing cells. The monocyte- and T cell-depleted fraction was layered on hypertonic metrizamide gradients (analytical grade, 14·5%; Nycomed, Oslo, Norway) and the cells at the

interface were collected. After washing in less hypertonic solution to regain isotonicity, the cells were treated with a MoAb to CD45RA (Leu-18; Becton Dickinson, San Jose, CA) for 45 min at 4°C and panned on a mouse γ-globulin-coated culture dish, and finally the non-adherent cells were collected as a DC-enriched population.

Enrichment of DC by the use of cytokines

This was done exactly as described by Romani *et al.* [18]. PBMC were collected from heparinized fresh blood by centrifuging on a column of Ficoll–Conray. PBMC (5–20 10^6) were suspended in 3·0 ml of RPMI 1640 (Nipro, Osaka, Japan) plus 10% fetal calf serum (FCS; Filtron Pty Ltd, Brooklyn, Australia) and adhered on a plastic surface for 2 h, after which the non-adherent cells were washed out by gentle washing and the adherent cells were cultured for an additional 7 days with granulocyte-macrophage colony-stimulating factor (GM-CSF; 800 U/ml; Genzyme, Cambridge, MA) and IL-4 (500 U/ml; Genzyme). The addition of IL-4 and GM-CSF reproducibly gave growing DC aggregates at 5–7 days, which were dislodged by gentle pipetting.

The DC populations were confirmed by their typical dendritic morphology, high expression of surface markers such as HLA-DR, CD86 and CD80, and absence of other lymphoid markers. Moreover, the stimulatory capacity of these DC populations was confirmed in allogeneic MLR.

Allogeneic MLR

Graded doses of irradiated dendritic cells (40 Gy; Hiltex Co., Osaka, Japan) and graded doses of T cells were cultured in 200 μl of culture medium in 96-well culture plates (Corning Glass Works, Corning, NY) for 5 days at 37°C in a humidified incubator containing 5% CO_2. ^{3}H-TdR, at a dose of 5·0 μCi/ml (Amersham Life Science, Aylesbury, UK) was added to each well 12 h before the end of the culture and the cells were harvested onto glassfibre filter paper (LM101-10; Futaba Medical Co., Tokyo, Japan) using an automated harvester (LM-101; Futaba Medical Co.) and cell-bound radioactivity was counted in a liquid scintillation counter (Beckman LS 6500; Beckman Instruments, Inc., Fullerton, CA). The results were expressed as mean ct/min from five separate wells. In some of the experiments, 2 10^5 T cells and 1 10^4 DC were cultured for 5 days, centrifuged and the clear supernatants were preserved for estimating cytokines and NO.

Role of NO inhibitor on allogeneic MLR

In order to see the impact of NO inhibitor on the stimulatory capacity of DC in PBC, DC (1 10^4 cells) were isolated from five patients with PBC by cytokine enrichment method and T cells (2 10^5) were isolated from the normal volunteer and cultured in allogeneic MLR without or with 500 μM of N^G-monomethyl-L-arginine monoacetate (L-NMMA), a known inhibitor of NO (Wako). The proliferation of normal volunteer-derived T cells in response to allogeneic DC from PBC without or with L-NMMA was estimated after the culture period of 5 days. The supernatants from allogeneic MLR without or with L-NMMA were collected after the culture period and the concentrations of NO were measured using a NO estimation kit.

Production of NO and cytokines by DC

Culture for production of NO by DC was done according to a previous report [21,22] with slight modifications. In short, DC (1 10^6/ml), enriched from patients with PBC, CH-C and normal controls by cytokines, were cultured in RPMI 1640 plus 10% FCS for 48 h in the presence of *Staphylococcus aureus* Cowan I strain (SAC) (Pansorbin, no. 507861; Calbiochem, La Jolla, CA). After the end of culture, supernatants were collected, centrifuged and the production of NO and cytokines in the culture supernatants was estimated.

Expression of HLA-DR and CD86 on DC

The levels of expression of HLA-DR and CD86 on DC were analysed by direct methods using FITC-conjugated mouse anti-human MHC class II antigens (HLA-DR) (clone, Immu-357; Immunotech, Marseille, France) or PE-conjugated mouse anti-human CD86 (clone, 2331 (FUN-1); Pharmingen, San Diego, CA), respectively. The cells were washed and resuspended in a solution of PBS, 0·1% bovine serum albumin (BSA), and 0·1% sodium azide with saturating amounts of the respective MoAbs or isotype-matched irrelevant controls for 30 min at 4°C, after which the cells were washed twice and suspended in fresh buffer. DC populations were phenotyped with the above antibodies and analysed on a FACSCalibur (Becton Dickinson). The dead cells and contaminating lymphocytes were excluded by forward and side scatter properties. The levels of expression of HLA-DR and CD86 were shown as mean fluorescence intensities (MFI).

Estimation of IL-10 and IL-12

IL-10 and IL-12 in the supernatants were estimated by an ELISA method using commercial kit (IL-10 and IL-12 estimation kit; BioSource International, Camarillo, CA) according to the instructions of the manufacturer. Samples were incubated on microtitre plates coated with the respective MoAbs, followed by addition of a biotinylated second antibody. After removal of excess antibody, colour development was finished by enzymatic reaction of streptavidin peroxidase, the intensity of which was directly proportional to the concentration of the respective cytokines in the samples. The amounts of cytokines in the samples were estimated by calibrating the optical density (OD) values of the samples with the OD values of the standards, supplied with the kits using an ELISA reader (SJeia, Auto Reader, ER-8000; Sanko Junyaku Co., Tokyo, Japan). The lowest levels of cytokines detectable by these kits were IL-10 > 15·0 pg/ml, IL-12 > 7·8 pg/ml.

Estimation of NO

NO in the samples was estimated by assaying the stable end product NO_2^- by Griess reaction using a commercial kit (Griess Assay Kit NO kit-C; Wako), as described [21,22]. Aliquots of culture supernatants were incubated with 100 μl of Griess reagent (1% sulfanilamide, 0·1% nephthylethylenediamine, dihydrochloride, and 2·5% H_3PO_4) at room temperature for 10 min. The colour developed due to enzymatic reaction was estimated by an ELISA reader at 540 nm. Concentrations of NO in the samples were calibrated with a reference standard of sodium nitrite supplied with the kit and the levels of NO were expressed as μM.

Statistical analysis

Data were expressed as mean ± s.e.m. The statistical analyses were done by unpaired or paired *t*-test, when indicated. In addition, Welch's *t*-test or Mann–Whitney *U*-test were done when unpaired *t*-test was not indicated and Wilcoxon rank sum test was done when paired *t*-test was not indicated. $P < 0.05$ was considered statistically significant.

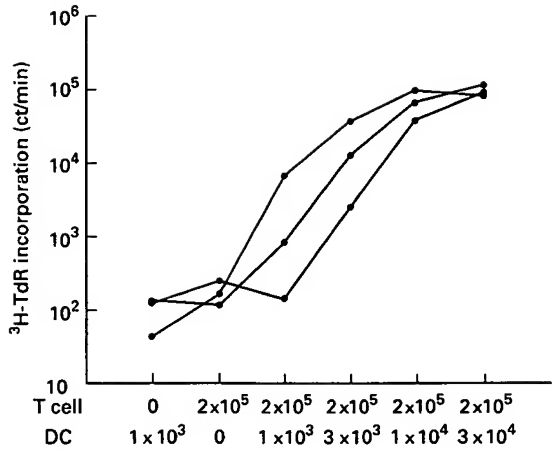


Fig. 1. Strong stimulatory capacity of dendritic cells (DC) in allogeneic MLR. DC and T cells were isolated, as described in Patients and Methods. The proliferation of allogeneic T cells (2 10^5 cells/well) in allogeneic MLR containing graded doses of irradiated DC (40 Gy) was estimated from the incorporation of ^{3}H-TdR (shown as ct/min) during the last 12 h of 5 days of culture. The culture containing only T cells showed incorporation of ^{3}H-TdR at < 250 ct/min. Three representative blastogeneses in allogeneic MLR showed that there were increased levels of ^{3}H-TdR incorporation as the number of DC increased up to 1 10^4/well.

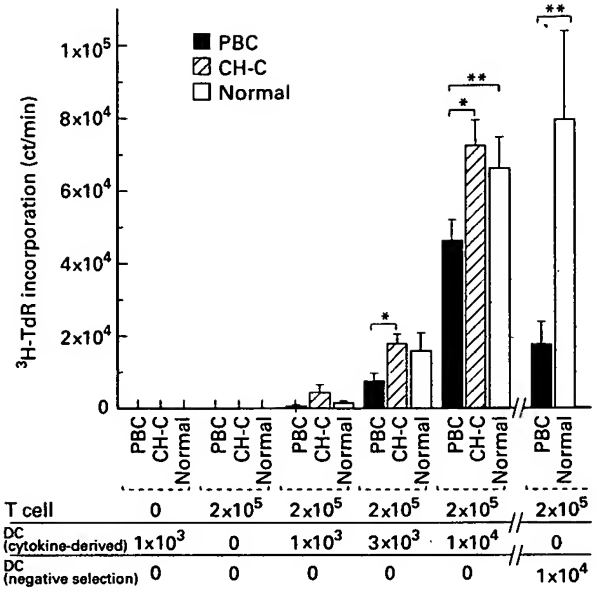


Fig. 2. Decreased blastogenesis in allogeneic MLR cultures containing dendritic cells (DC) from patients with PBC. DC were enriched from peripheral blood either by cytokines (cytokine-derived) or by negative selection method (negative selection). T cells were collected from one single normal volunteer. CH-C, Chronic hepatitis type C; normal, normal controls. The proliferation of T cells (2 10^5 cells/well) from normal volunteer in response to graded doses of irradiated DC from PBC, CH-C and normal controls was estimated from the incorporation of ^{3}H-TdR (shown as ct/min) during the last 12 h of 5 days of culture and is shown as mean ± s.e.m. The stimulatory capacity of DC was significantly lower in PBC compared with that from patients with CH-C and normal controls: $^*P < 0.01$; $^{**}P < 0.05$.

RESULTS

Morphologically, the isolated DC populations were identified by the presence of thin motile cytoplasmic processes or veils by phase contrast microscopy. These DC were negative for markers of other cells, e.g. CD3/19/21. Strong expression of HLA-DR was found on DC populations. Two methods were employed to isolate DC from peripheral blood. The negative selection method, in which DC were enriched by depleting other cell populations, showed 60–80% cells with features of DC, but the cell yield was comparatively low (1 10^5 DC/100 ml of peripheral blood). On the other hand, DC enriched by cytokines showed about 80–90% cells with dendritic morphology and the cell yield was considerably higher (2 10^6 cells/100 ml of blood). The functional capacity of the isolated DC populations was checked in allogeneic MLR. In order to discover an optimum condition, 2 10^5 T cells and graded doses of irradiated DC were cultured for 5 days and incorporation of ^{3}H-TdR for the last 12 h of culture was estimated as ct/min. Figure 1 shows the blastogenesis in allogeneic MLR from three preliminary experiments using 2 10^5 T cells and graded doses of DC. Culture containing only pure populations of T cells or only DC showed insignificant levels of blastogenesis and the background proliferation was <250 ct/min and <200 ct/min for T cells and DC, respectively. When the same T cells and irradiated DC were mixed and cultured for 5 days, however, there was a vigorous proliferation of T cells. Although the levels of blastogenesis increased along with the increase in number of DC in culture, the optimum level of T cell proliferation was recorded when 2 10^5 T cells and 1 10^4 DC were cultured in 200 μl in a 96-well culture plate.

As the experiments regarding the isolation of DC, their morphologic characterization, expression of surface antigens, and their functional capability to stimulate allogeneic MLR could be reproduced in several experiments, a series of experiments was undertaken to characterize the function of DC in PBC.

Low stimulatory capacity of DC in PBC

Figure 2 shows the incorporation of ^{3}H-TdR in allogeneic MLR containing irradiated T cells from a normal volunteer and DC from patients with PBC, CH-C and normal controls. Although the numbers of T cells were the same (2 10^5 cells/well) in all allogeneic MLR cultures, the levels of blastogenesis in allogeneic MLR increased as the numbers of DC were increased in all three diagnostic conditions. DC were enriched either by using cytokines or by negative selection methods, as described in Patients and Methods. The levels of blastogenesis (shown as ct/min) were significantly lower in allogeneic MLR containing DC (1 10^4/well) from patients with PBC compared with normal controls, irrespective of the method of isolation of DC ($P < 0.05$). Moreover, the levels of blastogenesis were significantly lower in PBC compared with CH-C when 3 10^3 and 1 10^4 DC/well were used in allogeneic MLR ($P < 0.01$).

Lack of correlation between clinical data and ct/min in allogeneic MLR

We could not find any correlation between the level of blastogenesis and the levels of alamine transaminase (ALT), aspartate transaminase (AST) or alkaline phosphatase (ALP), either in

Table 2. Cytokine production in allogeneic MLR and by dendritic cells (DC)

Dendritic cells*	Allogeneic MLR supernatant IL-10 (pg/ml)	IL-12 (pg/ml)	DC supernatant† IL-12 (pg/ml)
PBC	27·5 ± 8·3‡ (13)§	65·0 ± 20·8 (11)	132·9 ± 75·1 (7)
CH-C	31·9 ± 9·0 (11)	111·9 ± 51·0 (10)	173·3 ± 96·0 (6)
Normal controls	29·9 ± 12·9 (8)	61·1 ± 21·9 (7)	237·0 ± 105·6 (5)

* Dendritic cells (1 10^4/well) from patients with PBC, chronic hepatitis C (CH-C) and normal controls were cultured in allogeneic MLR with T cells (2 10^5/well) from a normal volunteer for 5 days, as described in Patients and Methods.

† Pure populations of DC (1 10^6 cells/ml) from patients with PBC, CH-C and normal controls were cultured for 48 h with *Staphylococcus aureus* Cowan I strain. The supernatants were collected and the levels of IL-10 and IL-12 were measured by ELISA and expressed as pg/ml (mean ± s.e.m.)‡.

§ Number of cases. There was no significant difference in the levels of IL-10 and IL-12 in any diagnostic condition.

PBC or in CH-C. The histopathological staging of liver cirrhosis in PBC did not show any relationship with the level of blastogenesis (data not shown).

Surface markers on DC

The fluorescence intensity of representative DC from six cases of each experimental group was assayed on two occasions. The DC from a normal volunteer who donated T cells in all experiments were taken as a positive control to standardize quantification between the two occasions. The levels of expression of HLA-DR on DC from PBC (shown by MFI) were 105 ± 28 (mean ± s.e.m.) and were not significantly different from the MFIs of HLA-DR in CH-C (132 ± 28) and normal controls (126 ± 28) ($P > 0·1$). The expression of CD86 was also not significantly different between PBC (165 ± 16), CH-C (131 ± 63) and normal controls (197 ± 34) ($P > 0·1$).

Cytokine production in allogeneic MLR and by DC

The levels of IL-10 and IL-12 in allogeneic MLR supernatants and IL-12 produced by DC are shown in Table 2. There was almost no difference in IL-10 levels in allogeneic MLR supernatants containing DC from PBC (27·5 ± 8·3 pg/ml), CH-C (31·9 ± 9·0 pg/ml), and normal controls (29·9 ± 12·9 pg/ml). The levels of IL-12 were also not different between PBC (65·0 ± 20·8 pg/ml), CH-C (111·9 ± 51·0 pg/ml), and normal controls (61·1 ± 21·9 pg/ml).

IL-12 produced by DC from patients with PBC (130·9 ± 75·1 pg/ml) was lower than that from patients with CH-C

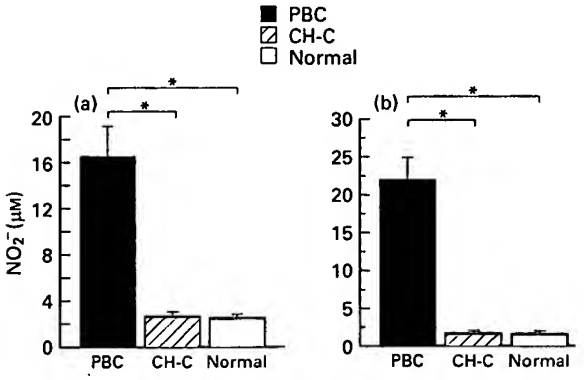


Fig. 3. Increased NO production by dendritic cells (DC) from patients with PBC. T cells (2 10^5/well) from a normal volunteer and DC (1 10^4/well) from patients with PBC, chronic hepatitis type C (CH-C) and normal controls were cultured for 5 days in allogeneic MLR (a). Pure populations of DC (1 10^6/ml) isolated by the cytokine enrichment method were cultured with *Staphylococcus aureus* Cowan I strain (SAC; 0·0075% w/v) for 48 h (b). NO in the culture supernatants was estimated by colourimetric assay using Griess's method and the amounts of NO were shown as μM (mean ± s.e.m.). Significantly higher amounts of NO were produced in allogeneic MLR containing DC from PBC compared with allogeneic MLR containing DC from CH-C and normal controls. Pure cultures of DC from PBC also produced significantly higher amounts of NO compared with the DC from CH-C and normal controls. *$P < 0·001$.

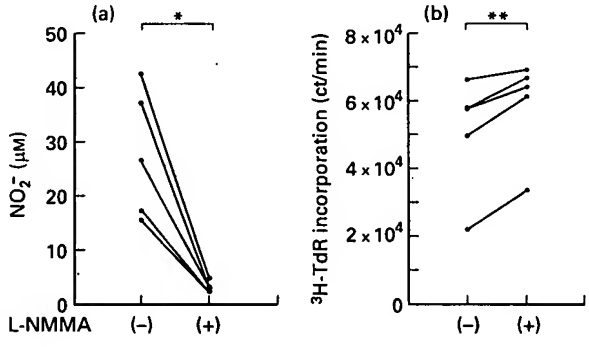


Fig. 4. Role of NO inhibitor in the production of NO and blastogenesis in allogeneic MLR. T cells (2 10^5/well) from a normal volunteer and dendritic cells (DC; 1 10^4/well) from patients with PBC were cultured without (–) and with (+) 500 μM of N^G-monomethyl-L-arginine monoacetate (L-NMMA) in allogeneic MLR for 5 days. The levels of NO (a) in culture supernatants of allogeneic MLR were measured by Griess reaction using NO estimation kit and the levels of blastogenesis (b) were measured as incorporation of ^{3}H-TdR (shown as ct/min). The addition of L-NMMA caused a decrease in NO (a) and increase in blastogenesis (b) in all five cases with PBC. The mean level of NO was significantly lower (*$P < 0·01$) and the mean level of blastogenesis was significantly higher (**$P < 0·05$) in allogeneic MLR with L-NMMA compared with allogeneic MLR without L-NMMA.

(177·3 ± 96·0 pg/ml), and normal controls (237·0 ± 105·6 pg/ml), but the difference was not statistically significant.

Increased NO production by DC from PBC

As shown in Fig. 3a, the levels of NO in allogeneic MLR supernatant containing DC from PBC (16·3 ± 2·9 μM, $n = 16$) were significantly higher than the amounts of NO produced in allogeneic MLR containing DC from CH-C (2·7 ± 0·4 μM, $n = 10$) and normal controls (2·5 ± 0·3 μM, $n = 9$) ($P < 0·001$). The levels of NO were not dependent on the stages of cirrhosis. Again, we could not find any correlation between the level of NO and the levels of transaminases (data not shown). NO is mainly produced by APC and DC are supposed to be one of the main producers of NO in allogeneic MLR supernatant. To have direct evidence of increased NO production by DC we cultured DC with SAC, a known inducer of NO. DC from patients with PBC (21·9 ± 2·8 μM, $n = 11$) produced significantly higher amounts of NO compared with DC from patients with CH-C (1·6 ± 0·3 μM, $n = 5$) and normal controls (1·6 ± 0·3 μM, $n = 6$) ($P < 0·001$) (Fig. 3b).

Restoration of stimulatory capacity of DC using NO inhibitor

Increased production of NO and decreased blastogenesis were documented in allogeneic MLR containing DC from patients with PBC. In order to have more insight in this regard, we estimated the production of NO and blastogenesis in allogeneic MLR containing T cells from a normal volunteer and DC from five patients with PBC without or with the addition of L-NMMA, a known NO inhibitor. As shown in Fig. 4a, the addition of L-NMMA to allogeneic MLR culture resulted in a significant decrease of NO (3·0 ± 0·4 μM *versus* 27·8 ± 5·3 μM, with L-NMMA *versus* without L-NMMA; $P < 0·01$). There was a significant increase in the level of blastogenesis during allogeneic MLR when cultured in the presence of L-NMMA (59 500 ± 6495 ct/min *versus* 51 106 ± 7578 ct/min, with and without L-NMMA, respectively; $P < 0·05$) compared with blastogenesis in allogeneic MLR done without L-NMMA (Fig. 4b).

DISCUSSION

Allogeneic MLR, in which responder allogeneic T cells proliferate in response to stimulation by non-T cells, is commonly used to evaluate the stimulatory capacity of APC. Figure 2 shows that the level of blastogenesis in allogeneic MLR containing DC from patients with PBC was significantly lower than that in allogeneic MLR containing DC from patients with CH-C and normal controls. As described in Patients and Methods, DC were enriched from peripheral blood by two techniques: cytokine-derived and negative selection method. The low stimulatory capacity of DC in PBC was evident irrespective of the methodology used to isolate DC from peripheral blood. The decreased blastogenesis in allogeneic MLR containing DC from patients with PBC compared with DC from patients with CH-C and normal controls could be attributable neither to a functional defect of T cells nor to the influence of serum or other inhibitory factors in PBC [23], because T lymphocytes were collected from a single normal volunteer in all allogeneic MLR, autologous sera were never used in any experiment, and all cultures were done and maintained under almost identical conditions. The decreased stimulatory capacity of DC from PBC and the mostly unaltered APC function of DC in CH-C have shown the specific nature of dysfunction of DC in PBC and have ruled out the possibility that the reduced stimulatory capacity of DC in PBC might be part of a generalized dysfunction of DC in chronic liver diseases.

Our data regarding the reduced blastogenesis and defective function of DC are in agreement with what has been reported regarding the dysfunction of DC in most other autoimmune diseases, such as psoriasis, SLE and RA [14–16], but apparently do not support what James *et al.* [7] reported regarding the allogeneic MLR in PBC. We are not certain of the mechanism underlying this difference, but it might be due to the difference in experimental design. James *et al.* [7] used an equal number of T and non-T cells in allogeneic MLR, whereas based upon the findings of our preliminary experiments, we used at most 5% DC and 95% T cells in most of the experiments. Moreover, to optimize the influence of T cells on blastogenesis in allogeneic MLR, we used T cells from one normal volunteer in all allogeneic MLR, whereas James *et al.* performed allogeneic MLR using T cells and non-T cells from different individuals.

Although it is hard to explain the low autologous MLR [7,8] and decreased production of cytokines [10] in spite of the presence of increased numbers of activated T lymphocytes in PBC [8,12], data from our experiments showing the low stimulatory capacity of DC from PBC have formed the scientific basis of these observations. DC are the critical cells in autologous MLR [24] and DC/T cell clusters are the main source of cytokines during T cell activation. The low stimulatory capacity of DC from PBC might be responsible for low autologous MLR and decreased production of cytokines, because DC are critical cells for autologous MLR and cytokines are produced by DC/T cell clusters during an immune response, although future studies will be needed to clarify these points fully.

The mechanism underlying the defective function of DC in PBC was analysed by characterizing the expression of surface antigens on DC, estimating the production of immunoregulatory cytokines, such as IL-10 and IL-12, and exploring the capacity of DC to produce free radicals, such as NO.

The expression of surface antigens such as HLA-DR and CD86 was not significantly different between DC from PBC, CH-C or normal controls (see Results). The levels of IL-10 and IL-12 in supernatants from allogeneic MLR containing DC from PBC, CH-C and normal controls did not show any marked difference (Table 2). The amounts of IL-12 produced by DC from PBC were lower than those produced by normal controls, but the difference was not statistically significant ($P > 0·1$).

The amounts of NO in allogeneic MLR supernatant containing DC from PBC were significantly higher than the levels of NO in allogeneic MLR supernatant containing DC from CH-C and normal controls (Fig. 3a). NO in allogeneic MLR supernatant is supposed to be produced mostly by DC [21,22], but in order to have direct evidence of increased NO production by DC in PBC, we examined the levels of NO in pure DC culture. DC from normal controls and CH-C produced insignificant amounts of NO, but DC from PBC produced significantly higher amounts of NO (Fig. 3b). Increased NO produced by DC from PBC explained the low blastogenesis in allogeneic MLR, because NO has been reported to inhibit MLR [21,22]. In order to find more direct evidence of NO in low allogeneic MLR in patients with PBC, we examined NO production and blastogenesis in allogeneic MLR, either adding or not adding L-NMMA, a potent inhibitor of NO. In all five cases with PBC, addition of L-NMMA resulted in a significant decrease of NO (Fig. 4a) and a subsequent increase of blastogenesis in allogeneic MLR (Fig. 4b). Moreover, the level of blastogenesis in

allogeneic MLR containing DC from PBC and T cells from a normal volunteer with L-NMMA showed no statistical difference from the blastogenesis of allogeneic MLR containing DC from normal controls and CH-C and T cells from same normal volunteer. Thus, a direct relationship between the increased NO production by DC and breakdown of tolerance to autoantigen in PBC is highly predictable, because increased production of NO has been implicated in the pathogenesis of many autoimmune processes, such as ulcerative colitis [25], autoimmune destruction of β cells of pancreas [26], and in murine spontaneous autoimmune diseases [27]. Moreover, Langerhans cells, a type of DC, have been reported to trigger psoriatic disease processes by enhancing the production of NO and thus a relationship between the progression of autoimmune disease and NO production by DC has been established [14]. A relationship between the production of NO and a break in self tolerance is predictable, because NO has been reported to cause damage to healthy tissue by inhibiting various enzymes, in addition to the toxic effects of NO on microbes, parasites and malignant cells [28]. Moreover, the co-culture of islets cells with chemicals, which produce large amounts of NO also lead to cell death, whereas a NO inhibitor suppresses the destruction of islet cells [29]. Thus, NO might be implicated in autoimmune tissue destruction. Although increased production of NO by DC has been shown in PBC, it remains to be elucidated whether NO is produced secondary to tissue destruction in PBC or is related to the pathogenesis of PBC. The very insignificant level of NO produced by CH-C in our experiments and the reports showing reduced levels of NO in chronic hepatitis [30] exclude the possibility that damage of biliary epithelium or hepatocytes would be primarily responsible for increased levels of NO in PBC.

The experiments presented here showing the dysfunction of DC and increased production of NO by DC have contributed considerably to our understanding of the pathogenesis of PBC. The findings from these experiments, along with the report that ursodeoxycholic acid, a therapeutic tool in PBC, inhibits NO production by APC [31], have also inspired optimism that an improved therapeutic approach can be developed for PBC by modulating NO production by dendritic cells.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research (C) (no. 40243779, 1997–98) from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1 Kaplan MM. Medical progress: primary biliary cirrhosis. N Eng J Med 1987; **316**:521–8.
2 Culp KS, Fleming CR, Duffy J, Baldus WP, Dickson ER. Autoimmune associations in primary biliary cirrhosis. Mayo Clin Proc 1982; **57**:365–70.
3 Yamada G, Hyoudou I, Tobe K, Mizuno M, Nishihara T, Kobayashi T, Nagashima H. Ultrastructural immunocytochemical analysis of lymphocytes infiltrating bile duct epithelia in primary biliary cirrhosis. Hepatology 1986; 6:385–91.
4 Krams SM, Dorshkind K, Gershwin ME. Generation of biliary lesions after transfer of human lymphocytes into SCID mice. J Exp Med 1989; **170**:1919–30.
5 Polson RJ, Portmann B, Neuberger J, Calne RY, Williams R. Evidence for disease recurrence after liver transplantation for primary biliary cirrhosis. Gastroenterology 1989; 97:715–25.
6 Dee HJ, Storb R. Graft-versus-host disease; pathophysiological and clinical aspects. Annu Rev Med 1984; **35**:11–24.
7 James SP, Elson CO, Waggoner JG, Jones EA, Strober W. Deficiency of the autologous mixed leukocyte reaction in patients with primary biliary cirrhosis. J Clin Invest 1980; **66**:1305–10.
8 Lindor KD, Weisner RH, Dickson ER, Homburger HA. The autologous mixed lymphocyte reaction in primary biliary cirrhosis: analysis of activation and blastogenesis of autoreactive T lymphocytes. Hepatology 1988; **8**:1555–9.
9 Suou T, Civeira MP, Kanof ME, Moreno-Otero R, Jones EA, James SP. Defective immunoregulation in primary biliary cirrhosis: $CD4^+$, $Leu-8^+$ T cells have abnormal activation and suppressor function *in vitro*. Hepatology 1989; **10**:408–13.
10 Spengler U, Moller A, Jung MC *et al.* T lymphocytes from patients with primary biliary cirrhosis produce reduced amounts of lymphotoxin, tumor necrosis factor and interferon-γ upon mitogen stimulation. J Hepatol 1992; **15**:129–35.
11 Menendez JL, Giron JA, Manzano L, Garrido A, Abreu L, Albillos A, Durantez A, Alvarez-Mon M. Deficient interleukin-2 responsiveness of T lymphocytes from patients with primary biliary cirrhosis. Hepatology 1992; **16**:931–6.
12 Bjorkland A, Festin R, Mendel-Hartvig I, Nyberg A, Loor L, Totterman TH. Blood and liver-infiltrating lymphocytes in primary biliary cirrhosis: increase in activated T and natural killer cells and recruitment of primed memory T cells. Hepatology 1991; **13**:1106–11.
13 Steinman R. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol 1991; 9:271–96.
14 Morhenn VB. Langerhans cells may trigger the psoriatic disease process via production of nitric oxide. Immunol Today 1997; **18**:433–6.
15 Garcia-Cozar FJ, Molina IJ, Cuadrano MJ, Marubayashi M, Pena J, Santamaria M. Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients. Clin Exp Immunol 1996; **104**:72–79.
16 Waalen K, Thoen J, Forre O, Hovig T, Teigland J, Natvig JB. Rheumatoid synovial dendritic cells as stimulators in allogeneic and autologous mixed leukocyte reactions, comparison with autologous monocytes as stimulators. Scand J Immunol 1986; **23**:233–41.
17 Ballardini G, Fallani M, Bianchi FB, Pisi E. Antigen presenting cells in liver biopsies from patients with primary biliary cirrhosis. Autoimmunity 1989; **3**:135–44.
18 Romani N, Gruner S, Brang D *et al.* Proliferating dendritic cell progenitors in human blood. J Exp Med 1994; **180**:83–93.
19 Bianchi L, de Groote J, Desmet VJ *et al.* Acute and chronic hepatitis revisited; review by an international group. Lancet 1977; **ii**:914–9.
20 Freudenthal PS, Steinman RM. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc Natl Acad Sci, USA 1990; **87**:7698–702.
21 Lu L, Bonham CA, Chambers FG, Watkins SC, Hoffman RA, Simmons RL, Thomson AW. Induction of nitric oxide synthase in mouse dendritic cells by IFN-γ, endotoxin, and interaction with allogenic T cells. J Immunol 1996; **157**:3577–86.
22 Bonham CA, Lu L, Li Y, Hoffman RA, Simmons RL, Thomson AW. Nitric oxide production by mouse bone marrow-derived dendritic cells. Transplantation 1996; **62**:1871–7.
23 Nouri Aria KT, Lombard M, Williams R. High serum levels of CD8 antigen in primary biliary cirrhosis: a possible cause of suppressor cell dysfunction. Clin Exp Immunol 1991; **86**:140–4.
24 Inaba K, Granelli-Piperno A, Steinman RM. Dendritic cells induce T lymphocytes to release B-cells-stimulating factors by an interleukin-2 dependent mechanism. J Exp Med 1983; **158**:2040–57.
25 Lundberg JON, Hellstrom PM, Lundberg JM, Alving K. Greatly increased luminal nitric oxide in ulcerative colitis. Lancet 1994; **344**:1673–4.
26 Corbett JA, Mcdaniel ML. Does nitric oxide mediate autoimmune destruction of β-cells: possible therapeutic intervention in IDDM. Diabetes 1992; **41**:897–903.

27 Weinberg JB, Granger DL, Pisetsky DS *et al.* The role of nitric oxide in the pathogenesis of spontaneous murine autoimmune disease: increased nitric oxide production and nitric oxide expression in MRL-*lpr/lpr* mice, and reduction of spontaneous glomerulonephritis and arthritis by orally administered N^G-monomethyl-L-arginine. J Exp Med 1994; **179**:651–60.

28 Kolb H, Kolb-Bachofen V. Nitric oxide: a pathogenetic factor in autoimmunity. Immunol Today 1992; **13**:157–60.

29 Kroncke KD, Kolb-Bachofen V, Berschick B, Burkart V, Kolb H. Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation. Biochem Biophys Res Commun 1991; **175**:752–8.

30 Amaro MJ, Bartolome J, Pardo M, Cotonat T, Lopez-Farre A, Carreno V. Decreased nitric oxide production in chronic viral hepatitis B and C. J Med Virol 1997; **51**:326–31.

31 Hattori Y, Murakami Y, Hattori S, Kuroda H, Kasai K, Shimoda S. Ursodeoxycholic acid inhibits the induction of nitric oxide synthase. Eur J Pharamacol 1996; **300**:147–50.